SHOUT
SHARE
IT

7712-43
2

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE
MASS.

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JAMES CASE, University of California, Santa Barbara
JOHN W. GOWEN, Colorado State University
SALLY HUGHES-SCHRADER, Duke University
LIBBIE H. HYMAN, American Museum of Natural History
SHINYA INOUÉ, Dartmouth College
J. LOGAN IRVIN, University of North Carolina
L. H. KLEINHOLZ, Reed College
JOHN H. LOCHHEAD, University of Vermont
ROBERTS RUGH, Columbia University
WM. RANDOLPH TAYLOR, University of Michigan
CARROLL M. WILLIAMS, Harvard University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

VOLUME 127
JULY TO DECEMBER, 1964

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $3.75. Subscription per volume (three issues), $9.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1. August, 1964

Page

Annual Report of the Marine Biological Laboratory 1

Clark, Mary E.
Biochemical studies on the coelomic fluid of Nephtys hombergi (Polychaeta: Nephtyidae), with observations on changes during different physiological states 63

Kahn, Arnold J.
The influence of light on cell aggregation in Polysphondylium pallidum. 85

Kurup, N. G.
The intermolt cycle of an anomuran, Petrolisthes cinctipes Randall (Crustacea—Decapoda) 97

Lance, Joan
The salinity tolerances of some estuarine planktonic crustaceans 108

Rai, K. S.
Cytogenetic effects of chemosterilants in mosquitoes. II. Mechanism of apholate-induced changes in fecundity and fertility of Aedes aegypti (L.) 119

Runnström, J.
On some properties of the jelly coat in oocytes and mature eggs of sea urchins. A study of phase-dependent changes of metaplasmic layers in the cell surface 132

Schmidt-Koenig, Klaus
Sun compass orientation of pigeons upon displacement north of the Arctic Circle 154

Seliger, H. H., J. B. Buck, W. G. Fastie and W. D. McElroy
Flash patterns in Jamaican fireflies 159

Yost, Henry T., Jr., Robert M. Glickman and Laurence H. Beck
Studies on the effects of irradiation of cellular particulates. IV. The time sequence of phosphorylation changes in vivo 173

No. 2. October, 1964

Allen, M. Jean
Embryological development of the syllid, Autolytus fasciatus (Bosc) (Class Polychaeta) 187

Brown, Frank A., Jr., H. Marguerite Webb and Franklin H. Barnwell
A compass directional phenomenon in mud-snails and its relation to magnetism 206

Brown, Frank A., Jr., Franklin H. Barnwell and H. Marguerite Webb
Adaptation of the magnetoreceptive mechanism of mud-snails to geomagnetic strength 221

COOPER, E. L., W. PINKERTON AND W. H. HILDEMANN
Serum antibody synthesis in larvae of the bullfrog, Rana catesbeiana.. 232
GREEN, JONATHAN P.
Morphological color change in the fiddler crab, Uca pugnax (S. I. Smith) 239
GREGG, JOHN R., JANE J. MACISAAC AND MARY ANN PARKER
Anaerobic glycolysis in amphibian development. Homogenates...... 256
HARRY, HAROLD W., AND JEROME B. SENTURIA
The effect of nitrogen, oxygen and carbon dioxide in producing the distress syndrome in Taphius glabratus (Gastropoda, Pulmonata)........ 271
HOLLAND, NICHOLAS D., AND SISTER AQUINAS NIMITZ, O. P.
An autoradiographic and histochemical investigation of the gut mucopolysaccharides of the purple sea urchin (Strongylocentrotus purpuratus).. 280
KESSEL, MARGARET M.
Reproduction and larval development of Acmaea testudinalis (Müller). 294
LUTZ, PAUL E., AND CHARLES E. JENNER
Life-history and photoperiodic responses of nymphs of Tetragoneuria cynosura (Say).. 304
MENGEBIER, WILLIAM L., AND MARIE M. JENKINS
Succinoxidase activity in homogenates of Dugesia dorotocephala...... 317
SPIELMAN, ANDREW
The mechanics of copulation in Aedes aegypti...................... 324
ZIMMERMAN, ARTHUR M.
Effects of mercaptoethanol on the furrowing capacity of Arbacia eggs.. 345
Abstracts of papers presented at the Marine Biological Laboratory........ 353

NO. 3. DECEMBER, 1964

BARNES, ROBERT D.
Tube-building and feeding in the chaetopterid polychaete, Spiochaetopterus oculatus.. 397
BARTH, LESTER G., AND LUCENA J. BARTH
Sequential induction of the presumptive epidermis of the Rana pipiens gastrula.. 413
CASE, JAMES
Properties of the dactyl chemoreceptors of Cancer antennarius Stimpson and C. productus Randall.................................. 428
GROSS, WARREN J.
Trends in water and salt regulation among aquatic and amphibious crabs 447
MUN, A. M., AND E. R. BURNS
Donor-host cell interaction in homologous splenomegaly in the chick embryo.. 467
NOVICK, ALVIN, AND JUOZAS R. VAISNYS
Echolocation of flying insects by the bat, Chilonycteris parnellii....... 478
READ, KENNETH R. H.
The temperature-coefficients of ribonucleases from two species of gastropod molluscs from different thermal environments............... 489

SLÁMA, K.
Hormonal control of respiratory metabolism during growth, reproduction, and diapause in male adults of Pyrrhocoris apterus L. (Hemiptera) 499
WILLIAMS, CARROLL M., AND PERRY L. ADKISSON
Physiology of insect diapause. XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, Antherea pernyi 511
YOST, HENRY T., JR., STEWART S. RICHMOND AND LAURENCE H. BECK
Studies on the effects of irradiation of cellular particulates. V. Acceleration of recovery of phosphorylation by polyanions 526
ZIMMERMAN, ARTHUR M.
The effects of mercaptoethanol upon form and movement of Amoeba proteus 538

Vol. 127, No. 1 August, 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

Sixty-Sixth Report, for the Year 1963—Seventy-Sixth Year

I. Trustees and Executive Committee (as of August 16, 1963) 1
Standing Committees
II. Act of Incorporation 4
III. Bylaws of the Corporation 4
IV. Report of the Director 6
Addenda:
1. Memorials 8
2. The Staff 9
3. Investigators, Lalor, Lillie and Grass Fellows, and Students .. 14
4. Fellowships and Scholarships 27
5. Training Programs 28
6. Tabular View of Attendance, 1959–1963 29
7. Institutions Represented 29
8. Evening Lectures 31
9. Evening Seminars 32
10. Members of the Corporation 33
V. Report of the Librarian 55
VI. Report of the Treasurer 56

I. TRUSTEES

Gerard Swope, Jr., *Chairman of the Board of Trustees,* 570 Lexington Ave., New York 22, New York.

Arthur K. Parpart, *President of the Corporation,* Princeton University.

James H. Wickersham, *Treasurer,* 791 Park Avenue, New York 21, New York.

Philip B. Armstrong, *Director,* State University of New York, College of Medicine at Syracuse.

C. Lloyd Claff, *Clerk of the Corporation,* Randolph, Mass.

Copyright © 1964, by the Marine Biological Laboratory

EMERITI

William R. Amberson, Marine Biological Laboratory.
C. Lalor Burdick, The Lalor Foundation.
W. C. Curtis, 504 West Mount Avenue, Columbia, Missouri.
Paul S. Galtsoff, Woods Hole, Massachusetts.
E. B. Harvey, Woods Hole, Massachusetts.
Charles W. Metz, Woods Hole, Massachusetts.
M. H. Jacobs, University of Pennsylvania.
F. P. Knowlton, Syracuse University.
W. J. V. Osterhout, The Rockefeller Institute.
Charles Packard, Woods Hole, Massachusetts.
A. C. Redfield, Woods Hole, Massachusetts.
A. H. Sturtevant, California Institute of Technology.

TO SERVE UNTIL 1967

Lester G. Barth, Columbia University.
John B. Buck, National Institutes of Health.
Aurin M. Chase, Princeton University.
Seymour S. Cohen, University of Pennsylvania, School of Medicine.
Teru Hayashi, Columbia University.
Lewis Kleinholz, Reed College.
Albert I. Lansing, University of Pittsburgh.
S. Meryl Rose, Tulane University.

TO SERVE UNTIL 1966

F. A. Brown, Jr., Northwestern University.
F. D. Carlson, The Johns Hopkins University.
Sears Crowell, Indiana University.
W. D. McElroy, The Johns Hopkins University.
C. Ladd Prosser, University of Illinois.
E. A. Scharrer, Albert Einstein College of Medicine.
Albert Szent-Györgyi, Marine Biological Laboratory.
Wm. Randolph Taylor, University of Michigan.

TO SERVE UNTIL 1965

Eric G. Ball, Harvard Medical School.
D. W. Bronk, The Rockefeller Institute.
Mac V. Edds, Jr., Brown University.
Rudolf Kempton, Vassar College.
I. M. Klotz, Northwestern University.
Arnold Lazarow, University of Minnesota Medical School.
Morris Rockstein, Medical Research Building, University of Miami.
George Wald, Harvard University, Biological Laboratories.

TO SERVE UNTIL 1964

Arthur L. Colwin, Queens College.
E. G. Butler, Princeton University.
K. S. Cole, The National Institutes of Health.
S. Kuffler, Harvard Medical School.
C. B. Metz, Institute for Space Biosciences, Florida State University.
Roberts Rugh, College of Physicians and Surgeons.
G. T. Scott, Oberlin College.
E. Zwilling, Brandeis University.

STANDING COMMITTEES

Executive Committee of the Board of Trustees

Arthur K. Parpart, *ex officio, Chairman*
Gerard Swope, Jr., *ex officio*
James H. Wickersham, *ex officio*
Philip B. Armstrong, *ex officio*
Teru Hayashi, 1966
William D. McElroy, 1966
Eric G. Ball, 1965
Sears Crowell, 1965
Mac V. Edds, Jr., 1964
I. M. Klotz, 1964

The Library Committee

M. A. Lauffer, *Chairman*
Seymour S. Cohen
Keith Porter
Irving M. Klotz
C. Ladd Prosser
James Lash
Stanley Watson

The Apparatus Committee

Albert I. Lansing, *Chairman*
Clifford Harding
David Potter
Arnold Lazarow
William D. McElroy
L. I. Rebhun

The Supply Department Committee

Rudolf Kempton, *Chairman*
W. J. Adelman
George Scott
Sears Crowell
Walter Herndon
John Simmons, Jr.

The Instruction Committee

Teru Hayashi, *Chairman*
A. C. Clement
Bostwick Ketchum
Robert K. Crane
Roger O. Eckert
DeWitt Stetten

The Buildings and Grounds Committee

Edgar Zwilling, *Chairman*
Mac V. Edds, Jr.
J. W. Hastings
J. W. Green
Daniel Grosch
Morris Rockstein

The Radiation Committee

H. H. Seliger, *Chairman*
P. M. Failla
Walter Vincent
R. L. Greif
C. C. Speidel
Walter Wilson

The Research Space Committee

William D. McElroy, *Chairman*
Mac V. Edds, Jr.
Teru Hayashi
Edgar Zwilling

The Committee for Nomination of Officers

Mac V. Edds, Jr., *Chairman*
Eric G. Ball
William D. McElroy
Sears Crowell
Irving M. Klotz
Teru Hayashi

II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 15, 1963)

I. The members of the Corporation shall consist of persons elected by the Board of trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees *ex officio,* who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti,* who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that Trustees *Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected for a term of five years and shall serve until his successor is selected and qualified, except that such term shall not run beyond the Annual Meeting of the Board following his 65th birthday; candidates over the age of 65 shall be elected on an annual basis. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEE MEETING AUGUST 16, 1963—EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of ten members as follows: *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The President shall act as Chairman of the Executive Committee and the Chairman of the Board of Trustees as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by Law.

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its actions shall be reported to the Board of Trustees.

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations at the annual meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire. (Chairman of the Board, President, Director, Treasurer, and Clerk.)

IV. REPORT OF THE DIRECTOR

To: THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY

Gentlemen:

I submit herewith the report of the seventy-sixth session of the Marine Biological Laboratory.

1. *Bylaws*

At a special meeting of the Board of Trustees (August 15, 1963) changes in the bylaws were adopted creating a new position of Chairman of the Board to be responsible principally for overall planning and executive action in the business, financial, legal and public relations areas. The President is to be responsible for overall planning and executive administration in the scientific area. The President will normally be a scientist. The office of Vice-President was abolished as redundant. Terms for all of the officers are specified and the responsibilities of the Trustees are spelled out. At a subsequent meeting the composition and powers of the Executive Committee were established by resolution. These changes are incorporated in the bylaws as published in this report.

2. *Plant Development*

It was noted in the report last year that the Laboratory received a grant from the National Science Foundation for a boat to be constructed as a replacement of the *Nereis* which has been in operation since 1925. This new boat will be completed in September, 1964, is 40 feet long, has a 14-foot beam, and is powered by a 160-horse-power diesel engine.

An additional grant from the National Science Foundation provides funds for extensive alterations in the library including a relocation of the offices and service rooms of the librarians, refurbishing the stacks, an extension of the reading room, improved lighting and new furniture. Part of this work will be completed during the winter of 1963–64, the remainder the following winter. This same grant provides funds for laboratory modifications which will concentrate the special apparatus in one area and additional funds for a wharf in front of the main laboratory extending out parallel to the salt water intake dock in the same location as the former *Cayadetta* dock.

3. *Planning Committee*

At its Annual Meeting the Board of Trustees authorized the appointment by the Executive Committee of an *ad hoc* committee on long-range planning, particularly in reference to modern dormitory, housing and dining hall facilities. This committee was set up by the Executive Committee and presented a report to the Executive Committee at its mid-winter meeting. The report was accepted by the Board of Trustees and the committee was instructed to explore ways of funding its recommendations.

4. *Laboratory Attendance and Turnover*

Elsewhere in this report is a "Tabular View of Attendance, 1959–1963." It will be noted that there has been a gradual increase in the scientific population at the Laboratory, resulting in considerable measure through an increased number of research assistants.

The full-time research personnel in the Laboratory this past winter numbered fifty-three. They were housed in twenty-five laboratories. There were twenty-five transient workers at the Laboratory, thirteen engaged in research activities, twelve reading in the library.

It has sometimes been suggested that the Marine Biological Laboratory is a closed organization with little turnover in research personnel. A survey of the situation revealed that there will be 76 investigators and 20 library readers in residence at the Laboratory during the summer of 1964 who were not present in 1963, and 155 investigators and 34 library readers in 1964 who were not present in 1959.

The three graduate training programs—Gamete Physiology, Comparative Physiology and Neuromuscular Physiology—serve as instruments for personnel change. There were 35 trainees in these three programs in 1963, only three of them repeaters. The fellowship programs, Lalor and Grass, included ten fellows in 1963, non of them repeaters.

Obviously there is a continuous flow of new personnel into the Laboratory, insuring a wide opportunity in the use of its facilities.

5. *Personnel Changes*

Because of his appointment as a Career Professor, Dr. Clark P. Read had to resign as head of the training program in Invertebrate Zoology but continues his interest in this program as a Consultant. We are very fortunate in attracting another very able invertebrate zoologist, Dr. W. D. Russell Hunter, to head up this training program.

6. *Deaths*

We note with sorrow the deaths of two Trustees *Emeriti,* Dr. F. P. Knowlton and Dr. W. J. V. Osterhout. Dr. Knowlton was a member of the staff of the physiology course from 1909–1926, a Trustee from 1923–1945 and a Trustee *Emeritus* from 1945 until his death in 1963. Dr. Osterhout was a Trustee from 1918–1940 and then a Trustee *Emeritus* until his death on April 9, 1964.

1. MEMORIALS

LAWRASON RIGGS

by CHARLES PACKARD

Lawrason Riggs, former Treasurer and President of this Corporation, died in January, 1963. Few of us realized the extraordinary ability and wide range of interests of this quiet and unassuming man. He was a successful lawyer in New York City. He found time to carry on the duties of Treasurer from 1924 to 1942, and to serve in the same capacity for the Oceanographic Institution from 1930 to 1950. This he did with the help of one part-time secretary. His ability as Treasurer is shown by the fact that during the depression years of the 1930s the income from our Endowment Fund remained fairly constant.

When Dr. Lillie died, Mr. Riggs was unanimously chosen to be President of the Corporation. Those of us who were privileged to serve with him will always remember his deep devotion to the Laboratory, his sympathetic understanding of its problems and his wise advice.

In whatever capacity he served he was always asked to continue year after year. He was Warden and Senior Warden of his New York Church for 45 years, an officer of this Laboratory for 20 years and of the Oceanographic Institution for 20 years.

His interests outside the law and the Woods Hole laboratories were exceptional. They were summed up by his friend, the Rector of the Church of the Ascension, in these words: "His life was one adventure after another—climbing mountains, gazing at the stars through his new telescope, walking through strange lands, picking up odd bits of archaeological finds, sailing the waters from Woods Hole to everywhere. He was at home among the classics."

Indeed, during his last stay in the hospital he had beside him a well-worn copy of the Greek New Testament.

His deep affection for this Laboratory was shown when he announced his retirement as President. As he spoke of his long association with the Laboratory his voice broke, and in the middle of a sentence he said "I cannot speak further."

The Laboratory has lost a most valuable and devoted friend of many years.

REINHARD DOHRN

by Albert Szent-Györgyi

It is befitting to remember here a man, Reinhard Dohrn, who was taken from our rows by death this year, though he did not belong to the Woods Hole family of researchers. He belonged to the wider circle of marine biologists, among whom he played a leading role, less through his own research than by providing for the research of others, with his devotion to science, his ability of organization, and his very high human qualities.

He was the Director of the Zoological Station in Naples, founded by his father, Anton Dohrn. He saved his institution through the turmoil of political events. This he did, less by political cunning than by his sincerity, honesty, modesty and idealism. To me he was the embodiment of all the higher and lofty ideals that science and humanities stand for.

He kept, always, the most friendly relations with Woods Hole in a spirit of brotherhood and not of competition. He parted at an old age, had a grand life full of struggle and noble endeavor, as a man's life should be, so we can bear no grudge to fate, but, all the same, his parting is a great loss to us all and to marine biology. We lost a great and very good friend, and a grand human being.

2. The Staff

EMBRYOLOGY

I. Instructors

James D. Ebert, Director, Department of Embryology, Carnegie Institution of Washington, in charge of course.

Allison L. Burnett, Associate Professor of Biology, Western Reserve University.

James W. Lash, Assistant Professor of Anatomy, University of Pennsylvania.

A. A. Moscona, Professor of Zoology, University of Chicago.

Arthur B. Pardee, Professor of Biology, Princeton University.

John W. Saunders, Jr., Professor of Biology, Marquette University.

II. Laboratory Assistants

John J. Coffey, Department of Biology, The Johns Hopkins University.

Sidney B. Simpson, Jr., Department of Zoology, Tulane University.

III. Lectures

Ch. Devillers	Structure and appearance of marginal zone and bilateral symmetry.
Beatrice Mintz	Experimental analysis of differentiation in the mammalian egg.
N. T. Spratt, Jr.	Morphogenetic movements in the early chick embryo.
J. P. Trinkaus	Cellular basis of *Fundulus* epiboly.
Ch. Devillers	Gastrulation in *Fundulus*.
R. B. Crawford	Energetics of development in *Fundulus*.
R. Goss	Regeneration and transplantation of scales in *Fundulus*.
J. Oppenheimer	Contributions, actual and potential, of *Fundulus* to experimental embryology.
T. D. Brock	Properties of cell surface in microorganisms.
J. F. Albright	Some dynamic aspects of antibody production.
R. Auerbach	Spleen and thymus development in relation to immunity.
J. J. Kollros	Tissue responses to thyroid hormone in regeneration.
I. R. Konigsberg	Clonal analysis of myogenesis.
F. C. Steward	Development of single plant cells.
A. C. Clement	The mollusk-annelid pattern of development.
A. C. Clement	Analysis of development in *Ilyanassa*.
K. R. Porter	Developmental cytology I.
K. R. Porter	Developmental cytology II.
M. Glimcher	Isolation, identification and properties of enamel protein and the ultra-structural organization of mineral and organic components.
D. Mazia	Mechanisms of cell division.
E. Bell	Keratin biosynthesis and organization in feather development.
J. Gross	Collagen formation and destruction in morphogenesis.
S. Spiegelman	The sequential reading of the genome.
A. Monroy	Analysis of development in echinoderms.
S. Cohen	Sequential protein synthesis during phage infection.
J. W. Hastings	Rhythmic physiological events in *Gonyolax*.
H. Halvorson	The timing of enzyme synthesis during synchronous growth in yeast.
M. Pollock	Stability of extrachromosomal characters and their possible role in differentiation.
M. Sussman	Cellular differentiation in the slime molds.
W. Rutter	Differentiation of pancreatic cells.
A. Monroy	Analysis of development in echinoderms.
C. Levinthal	RNA, messenger, and the problem of differentiation.
C. Levinthal	The folding of a multichain enzyme and genetic complementation.
T. B. Fitzpatrick	The anatomy and physiology of pigmentation.
G. Szabó	
A. Pardee	Cell regulatory mechanisms II.
W. Vincent	Messengers, nucleoli and the anatomy of a ribosome.
F. H. Wilt	Characteristics of RNA synthesis in cleaving sea urchin eggs.
J. Collier	Fractionation of nucleic acid in *Ilyanassa* embryos.
R. S. Edgar	Developmental genetics I.
R. S. Edgar	Developmental genetics II.
D. Bodenstein	Nerve regeneration in insects.
G. Weissmann	Vitamin A, lysosomes, and metamorphosis.

PHYSIOLOGY

I. CONSULTANTS

MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania.
ARTHUR K. PARPART, Professor of Biology, Princeton University.
ALBERT SZENT-GYÖRGYI, Director, Institute for Muscle Research, Marine Biological Laboratory.
W. D. MCELROY, Director, McCollum-Pratt Institute, The Johns Hopkins University.

II. INSTRUCTORS

J. WOODLAND HASTINGS, Associate Professor of Biochemistry, University of Illinois, in charge of course.
E. A. ADELBERG, Professor of Microbiology, Yale University.
PHILIP E. HARTMAN, Associate Professor of Biology, The Johns Hopkins University.
HARLYN HALVORSON, Professor of Bacteriology, University of Wisconsin.
SHINYA INOUÉ, Professor of Cytology, Dartmouth College.
K. E. VAN HOLDE, Associate Professor of Physical Chemistry, University of Illinois.
FRED KARUSH, Professor of Microbiology, University of Pennsylvania School of Medicine.
ALEXANDER KEYNAN, Israel Institute for Biological Research.

III. LABORATORY ASSISTANTS

JOHN SPUDICH, University of Illinois.
CAROLYN VEEDER, Swarthmore College.

IV. LECTURES

H. K. SCHACHMAN	The architecture of proteins.
ROBERT LOFTFIELD	Comments on the current hypothesis of protein synthesis.
EMANUEL MARGOLIASH	Structure and evolution of cytochrome c.
ROD CLAYTON	Physical aspects of photosynthesis.
GEORGE WALD	The problem of visual excitation.
ROD CLAYTON	Primary photochemical reactions in photosynthesis.
WILLIAM HAGINS	Physical mechanisms in photoreception.
DAN MAZIA	Title to be announced.
SOL SPIEGELMAN	A comparison of information transfer in DNA and RNA genomes.

Joint Sessions of the Embryology and Physiology classes

MARTIN POLLOCK	Stability of extrachromosomal characters and their possible role in differentiation.
MAURICE SUSSMAN	Cellular differentiation in the slime molds.
A. MONROY	Analysis of development in echinoderms.
JOHN LAW	Biological transalkylation reactions.
HERMAN KALCKAR	Ectobiology in microorganisms and in mammalian cells.
HOWARD HOLTZER	Problems of cellular differentiation in cartilage and muscle.
WILLIAM ARNOLD	A possible role of conduction electrons in biology.
ALBERT SZENT-GYÖRGYI	A survey of biochemistry.
G. T. REYNOLDS	The use of the image intensifier in microscopic observations.
IRVIN ISENBERG	Factors in charge-transfer complexing.

MARINE BOTANY

I. Consultant

William Randolph Taylor, Professor of Botany, University of Michigan.

II. Instructors

Richard C. Starr, Professor of Botany, Indiana University, in charge of course.
Philip W. Cook, Department of Botany, University of California.
Walter R. Herndon, Professor of Botany, University of Tennessee.
Peter S. Dixon, Hartley Botanical Laboratories, University of Liverpool.

III. Laboratory Assistants

Charles F. Cleland, Department of Biological Sciences, Stanford University.
Joanne K. Solem, Department of Plant Pathology, Cornell University.

IV. Collector

Austin E. Brooks, Department of Botany, Indiana University.

V. Lectures

Luigi Provasoli	Problems in the development of culture media for algae.

INVERTEBRATE ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University.
Libbie H. Hyman, American Museum of Natural History.
Alfred C. Redfield, Woods Hole Oceanographic Institution.

II. Instructors

Clark P. Read, Professor of Biology, Rice University, in charge of course.
John Anderson, Professor of Zoology, Cornell University.
G. F. Gwilliam, Associate Professor of Biology, Reed College.
George Holz, Professor of Microbiology, College of Medicine at Syracuse.
W. D. Russell Hunter, Lecturer in Zoology, University of Glasgow.
Roger Milkman, Associate Professor of Zoology, Purdue University.
Richard C. Sanborn, Professor of Zoology, Purdue University.
Irwin W. Sherman, Assistant Professor of Zoology, University of California, Riverside.

III. Assistants

Stephen Brown, Ohio State University.
James McDaniel, University of Oklahoma.

IV. Lectures

All special lectures were conducted jointly with those of the training program in comparative physiology.

MARINE ECOLOGY

I. CONSULTANTS

John H. Ryther, Woods Hole Oceanographic Institution.
Eugene P. Odum, University of Georgia.
Edwin T. Moul, Rutgers University.

II. INSTRUCTORS

W. Rowland Taylor, Department of Oceanography and the Chesapeake Bay Institute, The Johns Hopkins University, in charge of course.
Otto H. Kinne, Biologische Anstalt Helgoland, Hamburg, Germany.
John D. Palmer, Department of Biology, University of Illinois, Chicago.
Howard L. Sanders, Woods Hole Oceanographic Institution.
Lawrence B. Slobodkin, Department of Zoology, University of Michigan.

III. LABORATORY ASSISTANT

Donald C. Gordon, University of Rhode Island.

IV. LECTURES

Stanley W. Watson	The biology of marine nitrifying organisms.
Gordon C. Riley	Organic aggregates in sea water and their ecological significance.
Talbot H. Waterman	Orientation and visual physiology.
F. A. Brown, Jr.	Biological rhythms.
H. H. Seliger	Measurement of light in bioluminescent organisms.
Edwin T. Moul	Zonation on rocky shores.
Gordon A. Riley	Zooplankton-phytoplankton interrelationships.
E. R. Baylor	Langmuir cells and plankton distribution.
M. R. Carriker	Systematic Ecology Program at MBL.
R. S. Scheltema	Ecology of sessile and benthonic marine invertebrate larvae.
Robert G. Conover	Zooplankton trophic relationships.
H. A. Turner	Suggested mechanisms for formation of aggregations of benthonic marine organisms.
E. L. Mills	Adaptive significance of life history features in an amphipod crustacean.
N. B. Marshall	Deep-sea fishes.
C. Ladd Prosser	Theoretical aspects of physiological adaptations.

THE LABORATORY STAFF

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenback, Manager, Supply Department
Irvine L. Broadbent, Office Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Mrs. Vivien R. Brown
Mrs. Florence S. Butz
Mrs. Marion C. Chase
Mrs. Karen M. Driscoll
Mrs. Judith A. Keck
Mrs. Vivian I. Manson
Mrs. Virginia M. Morehouse

LIBRARY

Mrs. V. Brandenburg
Miss Jane Fessenden
Mrs. L. Joseph
Albert K. Neal
Mrs. Doris T. Ricker

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Eldon P. Allen
Bernard F. Cavanaugh
Manuel P. Dutra
Stanley C. Eldredge
Gardner F. Gayton
Robert Gunning
Walter J. Jaskun
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Henry F. Potter
James S. Thayer
Robert H. Walker, Jr.

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Seaver R. Harlow
Lowell V. Martin
Miss Ruth Preller
Frank E. Sylvia

SUPPLY DEPARTMENT

Arno J. Bowden
Donald P. Burnham
David H. Graham
Mrs. E. Green
Robert O. Lehy
Robert M. Perry
Paul Shave
Bruno F. Trapasso
John J. Valois
Jared L. Vincent
Hallett S. Wagstaff

DINING HALL AND HOUSING

Robert T. Martin, Manager, Food Service
Mrs. Elizabeth Kuil, Supervisor, Dining Room
Mrs. Ellen T. Nickelson, Supervisor, Dormitories
Alan G. Lunn, Supervisor, Cottage Colony

3. Investigators; Lalor, Lillie and Grass Fellows; Students

Independent Investigators, 1963

Adelberg, Edward A., Chairman, Department of Microbiology, Yale University
Adelman, William J., Jr., Associate Professor of Physiology, University of Maryland
Aljure, Emilio, Research Fellow (Rockefeller Fellow, University of Cali, Colombia), Columbia University, College of Physicians and Surgeons
Allen, Robert D., Associate Professor of Biology, Princeton University
Anderson, John M., Professor of Zoology, Cornell University
Armstrong, Clay M., Senior Assistant Surgeon, National Institutes of Health
Armstrong, Philip B., Professor and Chairman, Department of Anatomy, State University of New York College of Medicine at Syracuse
Arnold, William A., Principal Biologist, Oak Ridge National Laboratory
Auclair, Walter, Assistant Professor of Zoology, University of Cincinnati
Austin, George M., Independent Investigator, University of Oregon Medical School
Austin, Colin Russell, Member of External Scientific Staff, Medical Research Council, Physiological Laboratory, Cambridge, England

Bang, Frederik B., Professor and Chairman of Department of Pathobiology, The Johns Hopkins University School of Hygiene and Public Health

Barth, L. G., Professor of Zoology, Columbia University

Bell, Eugene, Associate Professor of Biology, Massachusetts Institute of Technology

Bennett, Michael V. L., Associate Professor of Neurology, Columbia University

Bibring, Thomas, Visiting Assistant Professor, Vanderbilt University

Binstock, Leonard, Electronic Engineer (Instrumentation), National Institutes of Health

Bodian, David, Director, Department of Anatomy, The Johns Hopkins University School of Medicine

Bretthauer, Roger K., Postdoctoral Fellow, University of Wisconsin

Brinley, F. J., Jr., Assistant Professor of Physiology, The Johns Hopkins University School of Medicine

Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University

Bryant, S. H., Associate Professor of Pharmacology, University of Cincinnati

Bullock, Theodore H., Professor of Zoology, University of California, Los Angeles

Burch, Helen B., Associate Professor of Pharmacology, Washington University

Burke, Joseph A., Assistant Professor of Biology, Acting Chairman of the Department, Loyola College, Baltimore

Burnett, Allison L., Associate Professor of Biology, Western Reserve University

Bush, Louise F., Assistant Professor of Zoology, Drew University

Carlson, Francis D., Professor of Biophysics and Chairman of the Department, The Johns Hopkins University

Chaet, Alfred B., Professor of Biology, American University

Cheney, Ralph Holt, Professor of Biology (General Physiology), Brooklyn College

Child, Frank M., Assistant Professor of Zoology, University of Chicago

Claff, C. Lloyd, Treasurer and Investigator, Single Cell Research Foundation, Inc.

Clark, Arnold M., Professor of Biology, University of Delaware

Clark, Eloise E., Assistant Professor in Zoology, Columbia University

Clement, Anthony C., Professor of Biology, Emory University

Cole, Kenneth S., Chief, Laboratory of Biophysics, NINDB, National Institutes of Health

Collier, Jack R., Marine Biological Laboratory

Colwin, Arthur L., Professor of Biology, Queens College

Colwin, Laura Hunter, Lecturer, Queens College

Cook, Philip William, Assistant Professor of Botany, University of Vermont

Cooperstein, Sherwin J., Associate Professor of Anatomy, Assistant Dean of Medical School, Western Reserve University School of Medicine

Copeland, Eugene, Professor and Chairman of Department of Zoology, Tulane University

Cormack, D. H., Research Associate in Molecular Biophysics, Institute of Molecular Biophysics, Florida State University

Costello, Donald P., Kenan Professor of Zoology, University of North Carolina

Crane, Robert K., Professor and Chairman of the Department of Biochemistry, The Chicago Medical School

Crowell, Sears, Professor of Zoology, Indiana University

D'Alesandro, Philip A., Assistant Professor, The Rockefeller Institute

Davidson, Harold, Physical Science Aide, National Institutes of Health

Deal, William C., Jr., Assistant Professor, Michigan State University

Dettbarn, Wolf-Dietrich, Assistant Professor of Neurology, Columbia University, College of Physicians and Surgeons

Devillers, Charles, Professor, Faculté des Sciences, Sorbonne, Paris

Diecke, F. P. J., Associate Professor of Physiology, George Washington University, School of Medicine

Dixon, Peter Stanley, University Lecturer, University of Liverpool

Ebert, James D., Director, Department of Embryology, Carnegie Institution of Washington

Eckert, Roger O., Assistant Professor of Zoology, Syracuse University

Edds, M. V., Jr., Professor and Chairman of the Department of Biology, Brown University

Ehrenstein, Gerald, Physiologist, National Institutes of Health

Failla, Patricia McClement, Associate Biophysicist, Argonne National Laboratory

Fawcett, Don W., Hersey Professor of Anatomy and Head of the Department, Harvard University Medical School

Feder, Ned, Assistant Professor of Biology, Harvard University

Fox, Stephen S., Assistant Professor, University of Michigan

Freeman, Alan R., Fellow in Neurology, Columbia University

Fuortes, M. G. F., Head, Neurophysiology Section, NINDB, National Institutes of Health

Furshpan, Edwin J., Assistant Professor of Neurophysiology and Neuropharmacology, Harvard Medical School

Gainer, Harold, USPHS Fellow, Columbia University

German, James L., Assistant Professor, Rockefeller Institute

Gilbert, Daniel L., Physiologist, National Institutes of Health

Grant, Philip, Program Director, Developmental Biology, National Science Foundation

Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College

Grosch, Daniel S., Professor of Genetics, North Carolina State College

Gross, Paul R., Associate Professor of Biology, Brown University

Grundfest, Harry, Professor of Neurology, Columbia University

Gwilliam, Gilbert F., Associate Professor of Biology, Reed College

Hagins, William A., Research Physiologist, National Institutes of Health

Hagiwara, S., Professor of Zoology, Department of Zoology, Brain Research Institute, U. C. L. A.

Halvorson, Harlyn O., Professor of Bacteriology, University of Wisconsin

Harding, Clifford V., Associate Professor of Physiology, Columbia University, College of Physicians and Surgeons

Hartman, Philip E., Associate Professor of Biology, The Johns Hopkins University

Hastings, J. Woodland, Associate Professor of Biochemistry, University of Illinois

Hayashi, Teru, Professor of Zoology, Columbia University

Hegyeli, Andrew, Institute for Muscle Research, Marine Biological Laboratory

Henley, Catherine, Research Associate, University of North Carolina

Herndon, Walter R., Professor and Head of the Department of Botany, University of Tennessee

Hervey, John P., Senior Electronic Engineer, The Rockefeller Institute

Hessler, Anita Y., Research Associate, Marine Biological Laboratory

Hill, Robert Benjamin, Instructor in Physiology, Dartmouth Medical School

Holz, George G., Professor and Chairman of the Department of Microbiology, S. U. N. Y., Upstate Medical Center, College of Medicine

Hopkins, Charles Adrian, Lecturer in Parasitology, University of Glasgow; and Rice University

Hoskin, Francis C. G., Assistant Professor, Columbia University, College of Physicians and Surgeons

Hubbard, Ruth, Research Associate, Harvard University

Humphreys, Tom, NIH Postdoctoral Fellow, Massachusetts Institute of Technology

Hunter, W. D. Russell, University Lecturer in Zoology, University of Glasgow

Hwang, Joseph C., Research Associate, Columbia University

Inoué, Shinya, Professor and Chairman of the Department of Cytology, Dartmouth Medical School

Isenberg, Irvin, Institute for Muscle Research, Marine Biological Laboratory

Janoff, Aaron, Assistant Professor of Pathology, New York University School of Medicine

Johnsson, Ruth, Institute for Muscle Research, Marine Biological Laboratory

Josephson, Robert K., Assistant Professor, University of Minnesota

Kaley, Gabor, Assistant Professor of Pathology, New York University School of Medicine

Kaminer, Benjamin, Institute for Muscle Research, Marine Biological Laboratory

Kane, Robert E., Assistant Professor of Cytology, Dartmouth Medical School

Karush, Fred, Professor of Microbiology, University of Pennsylvania School of Medicine

Kempton, Rudolf T., Professor of Zoology, Vassar College

Kenyan, Alexander, Foreign Instructor in Physiology, Israel Institute for Biological Research

Kinne, Otto, Director and Professor, Biologische Anstalt Helgoland

Kleinholz, L. H., Professor of Biology, Reed College

Krassner, Stuart M., Guest Investigator, Postdoctoral Research Fellow, The Rockefeller Institute

Kuffler, Stephen W., Professor of Neurophysiology and Neuropharmacology, Harvard Medical School

Kusano, Kiyoshi, Research Associate, Columbia University; Tokyo Medical and Dental University

Lansing, Albert I., Professor of Anatomy and Chairman of the Department, University of Pittsburgh

Lash, James W., Assistant Professor of Anatomy, University of Pennsylvania School of Medicine

Laster, Leonard, Chief of Gastroenterology Unit, National Institute of Arthritis and Metabolic Diseases

Laufer, Hans, Assistant Professor of Biology, The Johns Hopkins University

Lazarow, Arnold, Professor and Head of the Department of Anatomy, University of Minnesota

Lerman, Sidney, Associate Professor of Ophthalmology and Assistant Professor of Biochemistry, University of Rochester School of Medicine and Dentistry

Levin, Jack, Fellow, Department of Medicine, The Johns Hopkins Hospital and University

Levine, Lawrence, Associate Professor of Biochemistry, Brandeis University

Levinthal, Cyrus, Professor of Biophysics, Massachusetts Institute of Technology

Levy, Milton, Professor and Chairman of the Department of Biochemistry, New York University College of Dentistry

Lewis, Herman W., Program Director, Genetic Biology, National Science Foundation

Liebman, Paul A., Research Fellow, University of Pennsylvania

Lochhead, John H., Professor of Zoology, University of Vermont

Loewenstein, Werner R., Associate Professor of Physiology, Columbia University

Lopez, Enrique, Research Associate, Columbia University; National Institute of Cardiology, Mexico

de Lorenzo, A. J., Director, Anatomical and Pathological Research Laboratories, The Johns Hopkins University School of Medicine

Love, Warner E., Associate Professor of Biophysics, The Johns Hopkins University

Mackie, G. O., Associate Professor, University of Alberta

Mandriota, Frank J., Fellow in Neurology, Columbia University

Lutwak-Mann, Cecilia, Principal Scientific Officer on the Staff of the Agriculture Research Council of Great Britain, Space Biosciences Institute, Florida State University

Mann, Thaddeus, Director, Agriculture Research Council, Unit of Reproduction, Physiology and Biochemistry, Cambridge, England, Space Biosciences Institute, Florida State University

Marsland, Douglas, Research Professor, Biology Department, New York University

Mateyko, G. M., Associate Professor of Biology, New York University

McCarthy, Elaine S., Fellow in Research in Neurophysiology, Grass Instrument Company

McElroy, W. D., Chairman, Department of Biology and Director, McCollum-Pratt Institute, The Johns Hopkins University

Metz, Charles B., Professor of Biology, Florida State University

Milkman, Roger Dawson, Associate Professor of Zoology, Syracuse University

Mohsen, T., Assistant Professor, Faculty of Science, Dakar University, Republic of Senegal

Monroy, Alberto, Professor of Comparative Anatomy, University of Palermo, Italy

Moore, John W., Associate Professor of Physiology, Chief, Laboratory of Cellular Neurophysiology, Duke University

Moore, Richard O., Professor of Biochemistry, Ohio State University

Moran, Joseph F., Jr., Assistant Professor of Biology, Russell Sage College

Moscona, A. A., Professor of Zoology, University of Chicago

Mullins, L. J., Professor of Biophysics, University of Maryland

Musacchia, X. J., Professor of Biology, Saint Louis University

Nace, Paul Foley, Professor, Molecular Biology Research Unit, McMaster University

Nakajima, Shigehiro, Visiting Fellow and Research Scholar; United Cerebral Palsy Fellow, Columbia University (University of Tokyo Medical School)

Nakamura, Yutaka, Research Associate, Columbia University (University of Tokyo)

Nasatir, Maimon, Assistant Professor in Botany, Brown University

Nelson, Leonard, Associate Professor of Physiology, Emory University

Norris, W. E., Jr., Professor of Biology, Chairman of Department, Southwest Texas State College

Nyborg, Wesley L., Professor of Physics, University of Vermont

Overton, Jane, Associate Professorial Lecturer, University of Chicago

Palmer, John D., Assistant Professor in Biology, University of Illinois

Pardee, Arthur B., Professor of Biology, Princeton University

Parpart, Arthur K., Professor and Chairman of the Department of Biology, Princeton University

Passow, Hermann, Professor in Physiology, Head of Department, 11. Physiologisches Institut d. Universität d. Saarlandes, Homburg, Saar, Germany

Person, Philip, Chief, Special Dental Research Laboratory, Veterans Administration Hospital, Brooklyn

Pollock, Martin Rivers, Senior Lalor Fellow, National Institute for Medical Research, London, N.W.7

Porter, Keith R., Professor of Biology, Harvard University

Potter, David D., Associate Professor in Neurophysiology and Neuropharmacology, Harvard Medical School

Prosser, Clifford Ladd, Professor of Physiology and Head of the Department, University of Illinois

Rabin, Harvey, Assistant Professor, The Johns Hopkins University School of Hygiene

Read, C. P., Professor of Biology, Rice University

Rebhun, Lionel I., Assistant Professor of Biology, Princeton University

Reuben, John P., Assistant Professor of Neurology, Columbia University

Reynolds, George T., Professor of Physics; Director, Elementary Particles and Cosmic Ray Laboratory, Princeton University

Rockstein, Morris, Professor of Physiology, University of Miami School of Medicine

Rose, S. Meryl, Professor of Anatomy, Tulane University

Rosenberg, Philip, Research Associate, Columbia University, College of Physicians and Surgeons

Rosenkranz, Herbert S., Assistant Professor of Microbiology, Columbia University, College of Physicians and Surgeons

Rugh, Roberts, Associate Professor of Radiology, Columbia University, College of Physicians and Surgeons

Rustad, Ronald C., Assistant Professor of Physiology, Florida State University

Saito, Nozomu, Visiting Fellow and Research Scholar, United Cerebral Palsy Fellow, Columbia University (Tokyo Medical and Dental University)

Sanborn, Richard C., Professor of Zoology, Purdue University

Sanders, Howard L., Research Associate, Woods Hole Oceanographic Institution

Satir, Peter, Instructor in Zoology and College Biology, University of Chicago

Sato, Hidemi, Assistant Professor, Dartmouth Medical School

Saunders, John W., Professor and Chairman of the Department of Biology, Marquette University

Schmeer, Sister M. Rosarii, Chairman of Biological Education, Archdiocese, New York City

Schuel, Herbert, USPHS Postdoctoral Fellow, Biology Division, Oak Ridge National Laboratory

Scott, Sister Florence Marie, Professor of Biology and Chairman of the Department, Seton Hill College

Scott, George T., Professor and Chairman of the Department of Biology, Oberlin College

Searls, Robert L., Assistant Professor, University of Virginia

Seliger, Howard H., Associate Professor, McCollum-Pratt Institute and Department of Biology, The Johns Hopkins University

Shemin, David, Professor of Biochemistry, Columbia University

Sherman, Irwin W., Assistant Professor of Zoology, University of California, Riverside

Shivers, Charles Alex, USPHS Postdoctoral Fellow, Florida State University

Sichel, F. J., Professor and Chairman of the Physiology and Biophysics Departments, College of Medicine, University of Vermont

Siddiqui, Wasim A., Guest Investigator, The Rockefeller Institute

Simmons, John E., Jr., Assistant Professor of Biology, Emory University

SJODIN, RAYMOND A., Associate Professor of Biophysics, University of Maryland
SLOBODKIN, L. B., Associate Professor of Zoology, University of Michigan
SPEIDEL, CARL C., Professor of Anatomy, University of Virginia
SPINDEL, WILLIAM, Professor of Chemistry, Rutgers University
STARR, RICHARD C., Professor of Botany, Indiana University
STEINHARDT, JACINTO, Professor of Chemistry, Georgetown University
STEPHENS, GROVER C., Associate Professor of Zoology, University of Minnesota
STEVENSON, J. ROSS, Jacques Loeb Associate in Marine Biology (Kent State University)
STOECKNIUS, WALTHER, Assistant Professor, The Rockefeller Institute
STRITTMATTER, PHILIPP, Associate Professor of Biochemistry, Washington University
STUNKARD, HORACE W., Research Associate, American Museum of Natural History
SUDAK, FREDERICK N., Assistant Professor of Physiology, Albert Einstein College of Medicine
SURGENOR, DOUGLAS M., Dean of the Medical School, Chairman of the Department of Biochemistry, State University of New York at Buffalo
SZABÓ, GEORGE, Associate Biologist in Dermatology, Massachusetts General Hospital, Associate in Anatomy, Harvard Medical School
SZENT-GYÖRGYI, ALBERT, Director and Chief Investigator, Institute for Muscle Research, Marine Biological Laboratory
SZENT-GYÖRGYI, ANDREW, Professor of Cytology, Dartmouth Medical School
TASAKI, ICHIJI, Acting Chief, Laboratory of Neurobiology, NIMH, National Institutes of Health
TAYLOR, ROBERT E., Associate Chief, Biophysics Laboratory, NINDB, National Institutes of Health
TAYLOR, WILLIAM RANDOLPH, Professor of Botany and Curator of Algae in the Herbarium, University of Michigan
TAYLOR, WALTER ROWLAND, Assistant Professor of Oceanography, The Johns Hopkins University
TOMIZAWA, HENRY H., Senior Research Associate, Associate Professor of Biochemistry, Fels Research Institute, Antioch College
TORCH, REUBEN, Associate Professor of Zoology, University of Vermont
TRAGER, WILLIAM, Associate Professor, The Rockefeller Institute
TRAVIS, DAVID M., Assistant Professor of Pharmacology, Therapeutics and Medicine, University of Florida
TRINKAUS, JOHN PHILIP, Associate Professor of Biology, Yale University
TROLL, WALTER, Associate Professor, New York University, Institute of Industrial Medicine
TWAROG, BETTY M., Assistant Professor, New York University Medical School
TWEEDELL, KENYON S., Associate Professor of Biology, University of Notre Dame
ULBRICHT, WERNER W., Assistant Professor of Physiology, Duke University
USHERWOOD, P. N. R., United Cerebral Palsy Fellow, Columbia University; Department of Zoology, University of Glasgow
VAN HOLDE, K. E., Associate Professor of Chemistry, University of Illinois
VASINGTON, FRANK D., Postdoctoral Fellow, The Johns Hopkins University
VILLEE, CLAUDE A., Associate Professor of Biological Chemistry, Harvard Medical School
DE VILLAFRANCA, GEORGE W., Associate Professor of Zoology, Smith College
VINCENT, W. S., Associate Professor of Anatomy, University of Pittsburgh
WALD, GEORGE, Professor of Biology, Harvard University
WEBB, H. MARGUERITE, Associate Professor of Biology, Goucher College
WEBER, MORTON M., Associate Professor, Saint Louis University School of Medicine
WERMAN, ROBERT, Professor of Psychiatry, Indiana University
WICHTERMAN, RALPH, Professor of Biology, Temple University
WIERCINSKI, FLOYD J., Associate Professor of Biology, Drexel Institute of Technology
WILBER, CHARLES G., Dean of the Graduate School and Professor of Biological Sciences, Kent State University
WILSON, WALTER L., Associate Professor of Physiology and Biophysics, University of Vermont
WITTENBERG, JONATHAN B., Associate Professor of Physiology, Albert Einstein College of Medicine
YANAGITA, TAME MASA, Professor in Animal Physiology at Ochanomizu University, Japan

Zimmerman, Arthur M., Assistant Professor, State University of New York, Downstate Medical Center

Lalor Fellows, 1963

Pollock, Martin Rivers, National Institute for Medical Research, London
Laufer, Hans, The Johns Hopkins University
Nasatir, Maimon, Brown University
Mohsen, T., University of Strasbourg, Republic of Senegal, Africa
Rosenkrantz, Herbert S., Columbia University, College of Physicians and Surgeons

Lillie Fellow, 1963

Devillers, Charles, Faculté des Sciences, Sorbonne, Paris

Grass Fellows, 1963

Bullock, Theodore H., University of California, Los Angeles
Bernstein, Theodore W., University of Cincinnati, College of Medicine
Liebman, Paul A., University of Pennsylvania
McCarthy, Elaine S., Biological Laboratories, Harvard University

Beginning Investigators, 1963

Alvarez, Jaime, Tulane University
Bauer, Gustav Eric, University of Minnesota
Belamarich, Frank A., State University of New York at Buffalo
Bernstein, Theodore W., University of Cincinnati College of Medicine
Burchill, Brower R., Florida State University
Chervin, Paul N., University of Vermont
Churchich, Jorge E., University of Illinois
Cohen, Lawrence B., Columbia University
Cohen, William David, Columbia University
Couture, Kenneth G., Tulane University
Eskridge, Rosemary W., State University of New York at Buffalo
Fisher, F. M., Jr., Rice University
Ganguly, Banku B., Serampore and Presidency College, Calcutta
Grant, Robert J., Columbia University
Green, Jonathan P., The Johns Hopkins University
Hostetler, Karl, Western Reserve University School of Medicine
Jackson, James A., Western Reserve University
Kanno, Yoshinobu, Tokyo Medical and Dental University
Kriebel, Mahlon E., University of Washington
Lange, Mary T., Marquette University
Lentz, Thomas L., Yale University School of Medicine
Leslie, Robert B., Nottingham, England
Lindall, Arnold W., University of Minnesota
Malkin, Leonard I., Brown University
Marchalonis, John, The Rockefeller Institute
Nakase, Yasukiyo, Kitasato Institute in Tokyo
Pfohl, Ronald John, Michigan State University
Rovainen, Carl M., Harvard University
Sanel, Frances T., Columbia University, College of Physicians and Surgeons
Srebro, Richard, National Institutes of Health
Takata, Mitsuru, Duke University
Takenaka, Toshifumi, National Institutes of Health
Watkins, Dudley Taylor, Western Reserve Medical School
Wheeler, Maynard B., Columbia University, College of Physicians and Surgeons

Research Assistants, 1963

Acquaviva, Patricia, Seton Hill College
Akin, John R., Tulane University
Akin, Gwynn Marie, Tulane University
Alfano, Josephine, Cornell University Medical College
Andrews, Peter M., American University
Arnold, John M., University of Minnesota
Arthur, Elizabeth Jean, Purdue University
Ashman, Robert F., Columbia University, College of Physicians and Surgeons
Avery, Patricia Palmer, Wheaton College
Baird, Spencer L., Woods Hole
Baker, Cynthia, Harvard Medical School
Ballantine, Thomas Van Ness, Princeton University
Barker, Kenneth Ray, University of Mississippi
Barnwell, Franklin H., Northwestern University
Blair, Grace L., University of Minnesota
Borgman, Martha, Scarsdale, New York
Bosler, Robert, Harvard Medical School
Bradbury, Jack, Reed College
Brodin, Arlen, University of Minnesota
Brooks, Austin E., Indiana University
Brown, Stephen C., George Washington University
Carlson, Blenda, Florida State University
Carlson, Paul E., University of Minnesota
Cassidy, Rev. Joseph D., North Carolina State College
Chen, Henry L., Harvard University
Churchich, Margarita, University of Illinois
Clark, Charlotte, Harvard University
Cleland, Charles F., Stanford University
Coffey, John J., The Johns Hopkins University
Corff, Sandra C., State University of New York, Downstate Medical Center
Crosby, Gale M., Brandeis University
Crouse, Frances W., Biologische Anstalt Helgoland, Germany
Das, Asit, University of Illinois
Dashe, Charles K., University of Chicago
Daugherty, Wayne F., Jr., The Johns Hopkins University
Davis, Erskine D., National Institutes of Health
Diehl, Fred A., Western Reserve University
Diehl, Norma A., Western Reserve University
Doane, Marshall, University of Maryland, School of Medicine
Doebeli, Robert, Harvard Medical School
Donohoe, Charlie W., National Institutes of Health
Dougherty, William J., Princeton University
Ezell, Stiles D., Jr., Bryn Mawr College
Fallon, John F., Marquette University
Findlay, Mildred M., Syracuse University
Fitzjarrell, Austin T., Tulane University
Fok, Y. B., University of Maryland
Fiur, Ellen, Brandeis University
Foran, Elizabeth H., Smith College
Ford, Lincoln E., University of Rochester, School of Medicine
Freeman, Gary, University of Chicago
Fuller, Cameron B., Hunter College
Fusari, Margaret H., Boston University
Galis, Anna, Columbia University, College of Physicians and Surgeons
Gedmintas, Dana, University of Chicago
Gersh, Frank S., Reed College

Gershfeld, Norman L., National Institutes of Health
Ghiradella, Helen, Cornell University
Gibbs, R. G., University of Wisconsin
Goldman, Robert D., Princeton University
Gordon, Donald C., Jr., University of Rhode Island
Gough, Harry M., Brown University
Grabnar, Miklavi, The Johns Hopkins University
Grabske, Robert, University of Kansas
Grebe, Stephen C., Columbia University
Gupta, Raj Kumari, University of Alberta
Habas, Linda B., University of Illinois
Hall, Susan C., Harvard University
Hammond, Constance, University of Pittsburgh
Hardie, Jon H., University of Illinois
Harris, Edward M., Duke University
Hauschka, Stephen Denison, The Johns Hopkins University
Hawrylka, Eugenia A., New York University
Hickman, James Craig, Oberlin College
Hines, Rosemary, Saint Louis University
Hollenberg, Robert D., Harvard Medical School
Holliday, Mary P., Seton Hill College
Horwitz, Barbara Ann, Emory University
Hufnagel, Linda A., University of Vermont
Hughes, Kent S., The Chicago Medical School
Imlay, Marc James, American University
Jones, Frances E., Columbia University
Kaminsky, Norman M., George Washington University
Kapica, Suzanne, Russell Sage College
Katz, George, Columbia University
Kieblich, Jacoba, University of Amsterdam
Kimball, Frances, Reed College
Kinser, Glenn W., Jr., Indiana University
Kloetzel, John A., The Johns Hopkins University
Krainin, James, Harvard Medical School
Kunisawa, Riyo, University of California
Lambson, Roger O., Tulane University
Landolt, Casper Dale, Texas Western College
Larsen, Katherine, University of Chicago
Leitner, Leo G., Jr., National Institutes of Health
Lentz, Judith P., Yale University
Lewis, Dorothy, Institute for Muscle Research
Lewis, Hazel, Institute for Muscle Research
Lin, James C. H., North Carolina State College
Livingston, Laurie, Goucher College
Lloyd, Samuel, Princeton University
Long, Cedric W., Princeton University
Lubben, Barbara H., St. John's University
Macnamara, Gael R., Columbia University, College of Physicians and Surgeons
Mangan, Jerome, University of Cincinnati
Maritato, Cynthia A., University of Pennsylvania
Martin, Star, Harvard Medical School
McDaniel, James Scott, University of Oklahoma
McElroy, Kristine, American University
McKenzie, Donald K., National Institutes of Health
McLaughlin, Jane A., Institute for Muscle Research
Miller, Richard L., University of Chicago
Mohit, Roberta, New York University School of Medicine
Moule, Margaret, McMaster University

Mulcare, Donald J., University of Notre Dame
Munday, John C., Jr., University of Illinois
Nicholls, John G., Harvard Medical School
Palka, John M., University of California at Los Angeles
Parr, Earl L., Columbia University
Pearlstein, Robert M., Institute for Muscle Research
Penicnak, A. John, University of Massachusetts
Perry, Barbara, Institute for Muscle Research
Petrie, Roy H., Vanderbilt University School of Medicine
Philpott, Delbert E., Institute for Muscle Research
Prestidge, Louise S., Princeton University
Quinn, Constance, Syracuse University
Ray, Patricia A., Seton Hill College
Reed, Ray, Indiana University Medical Center
Rew, Robert E., Miami University, Oxford
Richmond, Arthur Pratt, Boston University
Ries, James J., National Institutes of Health
Robbins, Norman, The Rockefeller Institute
Robison, George Alan, Western Reserve University
Rosen, Barry, Brown University
Rosenthal, Jean, Albert Einstein College of Medicine
Sandlin, Ronald A., National Institutes of Health
Sanger, Joseph William, Dartmouth Medical School
Schnitzler, Ronald M., University of Vermont
Schoepf, Claude, Columbia University
Schulkind, Nancy M., Smith College
Schwend, Mary J., Albert Einstein College of Medicine
Sharpless, T. K., Princeton University
Shaw, Charis, Tulane University
Shilling, Jane O., Mount Holyoke College
Siegal, Marian R., Brandeis University
Silberman, Lester, State University of New York, Downstate Medical Center
Silveira, Marina, University of Sao Paulo, Brasil
Simmons, Norwood N., National Institutes of Health
Simpson, Sidney B., Tulane University
Skehan, Philip J., Syracuse University
Smith, Arlan E. S., Harvard University
Smith, Robert Harlan, American University
Smith, Stephen D., Tulane University
Solem, Joanne K., Cornell University
Spear, Joseph F., Loyola College
Specht, Philip C., Syracuse University
Spudich, James A., University of Illinois
Spudich, John L., University of Illinois
Stahl, Ruth C., The Johns Hopkins University
Steady, Henry M., Yale University
Stern, Doris N., Florida State University
Stern, Samuel, Florida State University
Szent-Györgyi, Gyula, Institute for Muscle Research
Szent-Györgyi, Marta, Institute for Muscle Research
Tamm, Sidney L., The University of Chicago
Taylor, Ellen L., University of Vermont
Thornton, William E., Vanderbilt University
Tolis, Helen, Wesleyan University
Toomajian, Lee C., Smith College
Trotter, Robert T., University of Vermont
Uter, Aston, American University
Utsumi, Sayaka, University of Pennsylvania School of Medicine

Van Norman, E., Princeton University
Veeder, Carolyn, Swarthmore College
Virkar, R. A., University of Minnesota
Wasserman, Eleanor S., Brandeis University
Weinberg, Robert, Massachusetts Institute of Technology
Windsor, Mary Linda, The Johns Hopkins University
Williams, Daniel B., Western Reserve University
Woodside, Kenneth H., University of Rochester School of Medicine and Dentistry
Zeidenberg, Phillip, Columbia University, College of Physicians and Surgeons
Zwick, Martin, Massachusetts Institute of Technology

Library Readers, 1963

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Baylor, Martha B., Independent Investigator, Marine Biological Laboratory
Berne, Robert M., Professor of Physiology, Western Reserve University, School of Medicine
Butler, Elmer G., Henry Fairfield Osborn Professor of Biology, Princeton University
Chase, Aurin M., Professor of Biology, Princeton University
Clifford, Sister Adele, Professor of Biology, College of Mt. St. Joseph
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania
Davis, Bernard D., Head of the Department of Bacteriology and Immunology, Harvard Medical School
DuBois, Arthur B., Professor of Physiology, University of Pennsylvania School of Medicine
Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Eisen, Herman N., Professor and Chairman, Department of Microbiology, Washington University School of Medicine
Farmanfarmaian, A., Faculty of Medicine, Pahlavi University, Iran
Fries, E. F. B., Professor of Biology, City College of New York
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Ginsberg, Harold S., Professor and Chairman of the Department of Microbiology, University of Pennsylvania
Glusman, Murray, Assistant Professor, Columbia University
Green, James W., Professor of Physiology, Rutgers University
Green, Maurice, Associate Professor of Microbiology, Saint Louis University
Handler, Philip, James B. Duke Professor of Biochemistry and Chairman of the Department of Biochemistry, Duke University
Haugaard, Niels, Associate Professor of Pharmacology, University of Pennsylvania
Hiatt, Howard H., Assistant Professor of Medicine, Harvard University
Hurwitz, Charles, Assistant Professor of Microbiology, VA Hospital, Albany
Isselbacher, Kurt J., Chief, Gastrointestinal Unit and Assistant Professor of Medicine, Massachusetts General Hospital and Harvard Medical School
Jacobs, Merkel H., Professor *Emeritus,* University of Pennsylvania
Kaltenbach, Jane C., Assistant Professor of Zoology, Mount Holyoke College
Karnovsky, Manfred L., Professor of Biological Chemistry, Harvard University
Keosian, John, Professor of Biology, Rutgers, The State University
Klein, Morton, Professor of Microbiology, Temple University Medical School
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University
Lauffer, Max A., Andrew Mellon Professor of Biophysics, University of Pittsburgh
Leighton, Joseph, Professor of Pathology, University of Pittsburgh School of Medicine
Lineaweaver, Thomas H., III, Marine Biological Laboratory
Marks, Paul A., Associate Professor of Medicine, Columbia University, College of Physicians and Surgeons
McDonald, Sister Elizabeth Seton, Chairman of the Department of Biology, College of Mt. St. Joseph
Needleman, Saul B., Research Biochemist and Instructor in Biochemistry, Abbott Laboratories
Novikoff, Alexander B., Research Professor, Albert Einstein College of Medicine
Rapport, Maurice M., American Cancer Society Professor of Biochemistry, Albert Einstein College of Medicine

Rosenberg, Evelyn K., Associate Professor of Pathology, New York University Medical Center
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rowland, Lewis P., Assistant Professor of Neurology, Neurological Institute, Columbia University
Schenck, J. R., Associate Research Fellow, Abbott Laboratories
Schneiderman, Howard A., Professor and Chairman of the Department of Biology, Western Reserve University
Spiegel, Melvin, Associate Professor of Biology, Dartmouth College
Spirtes, M. A., Associate Professor of Pharmacology, Hahnemann Medical College
Stetten, DeWitt, Jr., Dean of the Medical School, Rutgers, The State University
Warner, Robert C., Professor of Biochemistry, New York University School of Medicine
Wainio, Walter W., Chairman of the Department of Physiology and Biochemistry, Rutgers, The State University
Weiss, Leon, Associate Professor of Anatomy, The Johns Hopkins University School of Medicine
Wheeler, George E., Assistant Professor of Biology, Brooklyn College
Wilson, Thomas Hastings, Associate Professor of Physiology, Harvard Medical School
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zacks, Sumner I., Assistant Professor of Pathology, Pennsylvania Hospital

Students, 1963

All students listed completed the formal course program, June 17–July 27. Asterisk indicates students completing Post-Course Research Program, July 28–August 31.

** Post-Course only.

ECOLOGY

*Aronson, Wendy Sue, Vassar College
*Buggeln, Richard G., University of Hawaii
Daniels, Charles I., Duke University
*Dexter, Deborah M., University of North Carolina
Feingold, Alan O., Swarthmore College
Gebelein, Conrad, The Johns Hopkins University
*Holzapfel, Christine, Goucher College
Knopf, Garry N., University of Colorado
Myers, Judith, Chatham College
O'Brien, James F., Fordham University
*Reed, Kenneth J., Cornell University
*Robinson, Mary I., University of Chicago
Steen, Rebecca H., Yale University
*Thayer, Gordon, Oberlin College
West, Mary Jane, University of Michigan
*Zusy, Dennis R., Northwestern University

EMBRYOLOGY

*Battikh, Hani K., Qousair, Homs, Syria
Berger, Hillard, Vanderbilt University
*Burdick, Morton L., The Johns Hopkins University
Cassens, Gloria A., Indiana University
David, John R., New York University, School of Medicine
*Davidson, Richard L., Western Reserve University
*Epstein, Michael J., University of Texas
*Hartline, Daniel, Harvard University
*Kimmel, Charles B., The Johns Hopkins University
*Kohne, David E., Purdue University
*McWhinnie, Dolores, Marquette University

*Patton, Dorothy E., University of Chicago
Pollack, Robert E., Brandeis University
*Reeder, Ronald H., Massachusetts Institute of Technology
*Shostak, Stanley, Brown University
Spring, Elinor J., Harvard Medical School
*Stackhouse, Hamilton Lee, University of Arizona
Steen, Trygve, Yale University
*Terman, Stanley A., Brown University
*Yuyama, Shuhei, Florida State University

BOTANY

Benade, Leonard E., George Mason College of University of Virginia
*Campi, James R., Indiana University
*Carefoot, John R., Indiana University
Clements, Susan D., Montana State College
*Costlow, Judson, Indiana University
Cox, Edmond R., Jr., Middle Tennessee State College
Hammack, Robert E., University of Alabama
*Harris, Denny O., Indiana University
*Hodge, Martha D., University of Michigan
Johnson, Charles W., Wabash College
Kehlenback, Mrs. Edna K., Syracuse University
*Knox, John, Drew University
*Liddle, Larry B., University of Illinois
Pertz, Anita R., City College of New York
*Richardson, William N., Earlham College
*Sawa, Takashi, University of Louisville
*Schofield, Edmund A., Jr., Clark University
Shen, Eugene, University of Texas
*Walne, Patricia L., University of Texas
Weinkauff, Ann Mary, Oberlin College

PHYSIOLOGY

*Barlow, Robert B., Jr., The Rockefeller Institute
*Bownds, Marlin D., Harvard University
*Cownan, Annamma, Marquette University
Connelly, Yvonne, Northwestern University
DeSa, Richard John, University of Illinois
*Elzinger, Marshall, University of Illinois
Fratantoni, Joseph, Cornell Medical College
*Garrick, Linda S., The Johns Hopkins University
*Grey, Robert D., Washington University
*Hahn, William E., Tulane University
*Harrison, Stephen C., Harvard University
*Haynes, Julian F., Western Reserve University
*Hilden, Shirley, Stanford University
Humphrey, Richard, The Johns Hopkins Hospital
*Jacobson, Eugene D., Walter Reed Army Institute of Research
*Jacklett, Jon W., University of Oregon
*Leichtling, Ben H., Northwestern University
Madrid, Felix, University of Miami
*Mittenthal, Jay E., The Johns Hopkins University
Nauman, Dorothy C., Harvard University
*Phillips, Allen T., Michigan State University
Ponticorvo, Laura, Columbia University
Saha, Kunal, Calcutta, India
*Sederoff, Ronald R., University of California, Los Angeles

*Shaper, Joel H., University of California
*Stephens, Raymond E., University of Pittsburgh
Trivus, Robert H., Hahnemann Medical College
*Tunis, Mary Jane, University of California
**Muesing, Richard

INVERTEBRATE ZOOLOGY

Barbour, Stephen D., Temple University
Benson, Woodruff W., Emory University
Brown, James H., Cornell University
*Busser, John H., University of Rhode Island
Carter, Eugenie D., Drew University
Cavicchi, Phyllis A., Mt. Holyoke College
Chamberlain, Elizabeth, American University
*Cheney, Sarah D., Earlham College
Connelly, Sister Mary Edward, St. Bonaventure University
*Craig, Nessly C., Reed College
Dabney, Michael, Oberlin College
Dalsheimer, George H., The Johns Hopkins University
DeSanto, Robert S., Columbia University
*Dillard, Walter L., Oklahoma University
Dominique, Sister M., University of Notre Dame
Dyson, Martha M., Oberlin College
Etzler, Marilynn, Washington University
Feder, Ned, Harvard University
Grossi, Joseph C., Jr., College of Steubenville
Imlay, Marc J., American University
Jenkins, Virginia, University of Massachusetts
Jones, Helen E., Cornell University
Karstadt, Myra L., University of California, Berkeley
Lee, Sandra, Vassar College
*Lesh, Georgia E., Western Reserve University
*Levandowsky, Michael, City College of New York
Levowitz, Amy, Queens College
Lloyd, Margaret C., Bryn Mawr College
*Long, Nancy D., University of California
MacNab, Ann K., Rice University
*Morean, Edith, University of Minnesota
Myren, Richard T., Cornell University
Pogany, Gilbert C., Tulane University
*Smith, Charles C., Oklahoma University
*Smith, Clare D., University of Arizona
Stadler, Joan, University of Michigan
Stern, Edward L., University of Chicago
*Summers, William C., University of Minnesota
Teitelbaum, Dorothy J., Columbia University
Vianney, Sister M. Jean, University of Notre Dame

4. FELLOWSHIPS AND SCHOLARSHIPS, 1963

Bio Club Scholarship:
Michael Levandowsky, Invertebrate Zoology Course
Anita Pertz, Botany Course

Gary N. Calkins Memorial Scholarship:
William Summers, Invertebrate Zoology Course

Lucretia Crocker Scholarship:
John R. Carefoot, Botany Course
Mary I. Robinson, Ecology Course

Edwin Linton Memorial Endowment of the
Washington and Jefferson College:
John Bushnell

E. G. Conklin Fund:

R. L. Davidson, Embryology Course
D. E. Kohne, Embryology Course
Dorothy Patton, Embryology Course

Turtox Scholarship Fund:

Patricia L. Walne, Botany Course

5. TRAINING PROGRAMS

Fertilization and Gamete Physiology Training Program

I. Instructors

C. B. Metz	Florida State University, in charge of program
C. R. Austin	Cambridge University
A. Monroy	University of Palermo
L. Nelson	Emory University

II. Lectures

C. A. Shivers	Immunological studies on fertilization in frogs
D. Grosch	Reproductive processes in insects
R. Sager	Studies on sexuality in Chlamydomonas
E. Adelberg	Bacterial conjugation
W. O. Nelson	Inhibition of spermatogenesis
M. Nasatir	Peculiar propagatory processes in plants
M. Olsen	Parthenogenetic development in chicken and turkey eggs
T. Mann	Relation between the androgenic and spermatogenic activity of the male gonad
I. W. Rowlands	Intraperitoneal insemination

Neurophysiology Training Program

I. Instructors

S. W. Kuffler	Harvard Medical School, in charge of program
D. D. Potter	Harvard Medical School
E. J. Furshpan	Harvard Medical School

(No lectures given—only seminars)

Comparative Physiology Training Program

I. Instructors

C. Ladd Prosser	University of Illinois, in charge of program
G. Kaley	New York University
A. Janoff	New York University
Lewis Kleinholz	Reed College
B. Zweifach	New York University

II. Assistants

John Munday	University of Illinois
Linda Habas	University of Illinois
Ash Das	University of Illinois

III. Lectures

*C. L. Prosser	Physiological adaptation Comparative physiology of muscle
*Lewis Kleinholz	Invertebrate endocrinology
Gabor Kaley	Renin-angiotensin systems in control of peripheral circulation
Aaron Janoff	Biochemistry of leucocytes
*Frank A. Brown, Jr.	Biological rhythms Reception of magnetic fields
*Eugene Copeland	Chloride- and other secreting cells
*Talbot Waterman	Arthropod vision
*W. H. Sawyer	Comparative biochemistry of pituitary polypeptides
Grover Stephens	Utilization of dissolved organic material by invertebrates
*Clark Read	Metabolism of endoparasites
Chester Hyman	Comparison of open and closed circulations
*Benjamin Zweifach	Contractile endothelial cells and control of microcirculation
G. de Villafranca	Properties of contractile proteins from invertebrate muscles
*John Kanwisher	Temperature tolerance of insects
T. H. Bullock	Properties of cardiac ganglia of crustaceans

* Seminars only. The others were lecture-discussions or these plus seminars.

6. TABULAR VIEW OF ATTENDANCE, 1959–1963

	1959	*1960*	*1961*	*1962*	*1963*
Investigators—Total	427	458	458	494	490
Independent	215	231	224	235	227
Under instruction	45	42	32	44	34
Library Readers	51	50	49	56	51
Research Assistants	116	135	151	159	178
Students—Total	134	122	130	121	124
Invertebrate Zoology	55	49	40	38	40
Embryology	23	20	21	20	20
Physiology	27	28	28	28	28
Botany	20	18	19	20	20
Ecology	15	13	22	15	16
Total Attendance	561	580	586	615	614
Less persons represented as both investigators and students	4	2	1	4	5
	557	578	585	611	609
Institutions Represented—Total	143	144	132	118	120
By investigators	98	83	107	81	83
By students	73	61	70	57	70
Schools and Academies Represented					
By investigators	2	5	3	3	4
By students	12	2		2	
Foreign Institutions Represented	38	14	28	31	21
By investigators	29	11	21	17	15
By students	9	3	7	14	6

7. Institutions Represented, 1963

Abbott Laboratories
Alabama, University of
Albert Einstein Medical School
American Museum of Natural History
American University
Antioch College
Middle Tennessee State College
Minnesota, University of
Montana State University
Mount Holyoke College
New York State University, College of Medicine at Brooklyn

Argonne National Laboratory
Arizona, University of
Brandeis University
Brooklyn College
Brown University
Bryn Mawr College
California, University of
Carnegie Institution of Washington
Chatham College
Chicago Medical School
Chicago, University of
Cincinnati, University of
City College of New York
Clark University
College of Mt. St. Joseph on the Ohio
College of Steubenville
Colorado, University of
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Cornell University
Cornell University Medical College
Dartmouth College
Dartmouth College Medical School
Delaware, University of
Drew University
Drexel Institute of Technology
Duke University
Earlham College
Emory University
Florida State University
Florida, University of
Fordham University
George Washington University, School of Medicine
Georgetown University
Goucher College
Hahnemann Medical School
Harvard University
Harvard University Medical School
Hawaii, University of
Illinois, University of
Indiana University
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University, School of Medicine
Kent State University
Louisville, University of
Loyola College
Marquette University
Maryland, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Miami, University of
Michigan State University
Michigan, University of
New York State University, College of Medicine at Syracuse
New York University, Bellevue Medical Center
New York University, School of Dentistry
New York University, Washington Square College
North Carolina State College
North Carolina, University of
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oberlin College
Ohio State University
Oklahoma, University of
Oregon, University of
Pennsylvania, University of
Pennsylvania Medical School, University of
Pittsburgh, University of
Princeton University
Purdue University
Queens College
Reed College
Rhode Island, University of
Rice University
Rochester, University of, School of Medicine
Rockefeller Institute
Russell Sage College
Rutgers University
Saint Bonaventure University
Saint Louis University
Seton Hill College
Single Cell Research Foundation, Inc.
Smith College
Southwest Texas State College
Stanford University
State University of New York at Buffalo
Swarthmore College
Syracuse University
Temple University
Tennessee, University of
Texas, University of
Tulane University
Vanderbilt University
Vermont, University of
Veterans Administration Hospital
Virginia, University of
Wabash College
Walter Reed Army Institute of Research
Washington University
Washington, University of
Washington and Jefferson College
Washington University Medical School
Western Reserve University
Western Reserve University, School of Medicine
Wisconsin, University of
Yale University

Foreign Institutions Represented

Faculté des Sciences, Paris
University of Liverpool, England
Seramport and Presidency College, Calcutta, India
Biologische Anstalt Helgoland, Germany
University of Alberta, Edmonton, Canada
Agricultural Research Council of Great Britain
Faculty of Science, Dakar University, Republic of Senegal, Africa
Kitasato Institute, Tokyo, Japan
Universität d'Saarlandes, Homburg/Saar, Germany
National Institute for Medical Research, London, England
Tokyo Medical and Dental University, Japan
Ochanomizu University, Japan
Pahlavi, University of, Shiraz, Iran
Embassy of the Syrian Arab Republic, Syria
McMaster University, Hamilton, Ontario
Physiological Laboratory, Cambridge, England
Edinburgh University, Edinburgh, Scotland
University of Glasgow, Scotland
Inst. de Biologia, Mexico
University of Palermo, Italy
University of Sao Paulo, Brasil

Supporting Institutions, Agencies, and Individuals

Abbott Laboratories
Associates of the Marine Biological Laboratory
Atomic Energy Commission
CIBA Pharmaceutical Products, Inc.
The Commonwealth Fund
Josephine B. Crane Foundation
Duke University
Eli Lilly and Company
Dr. and Mrs. David W. Gaiser
The Grass Foundation
Mr. and Mrs. William H. Greer, Jr.
Hoffman-LaRoche, Inc.
Mr. and Mrs. George F. Jewett, Jr.
The Lalor Foundation
National Institutes of Health
National Science Foundation
Office of Naval Research
The Rockefeller Foundation
Dr. and Mrs. Henry W. Sandeen
Schering Foundation, Inc.
Scientific American, Inc.
E. R. Squibb and Sons
Gerald Swope, Jr.
The Upjohn Company
Wallace Laboratories
James H. Wickersham

8. Friday Evening Lectures, 1963

July 5	Teru Hayashi Columbia University	Contractile proteins and movement
July 12	Theodore H. Bullock University of California at Los Angeles Alexander Forbes Lecturer at the MBL	Ways and means of integrating in nervous systems. I
July 15 Monday	Theodore H. Bullock	Ways and means of integrating in nervous systems. II
July 19	Lawrence B. Slobodkin University of Michigan	Experimental studies of population dynamics
July 26	Charles Devillers Université de Paris Faculté de Science (Sorbonne), France	Mechanics of gastrulation

August 2	
Arthur B. Pardee Princeton University	Regulation of biochemical reactions
August 9	
Elvin A. Kabat Columbia Presbyterian Medical Center	Immunochemical studies on blood group substances
August 16	
Martin R. Pollock National Institute for Medical Research, London, England Senior Lalor Fellow at the MBL	The physiology and evolution of bacterial penicillinase
August 23	
Philip Handler Duke University	The evolution of proteins

9. Tuesday Evening Seminars, 1963

July 2	Richard De Sa, J. W. Hastings and A. E. Vatter	The scintillon—a new type of biological particle: an intracellular crystal which emits bioluminescent flashes
	James Spudich and J. W. Hastings	The inhibition of the bioluminescent oxidation of reduced flavin mononucleotide by 2-decenal
	Leonard Nelson	Spermatozoan cholinesterase
July 9	Warner E. Love and Nevenka M. Rumen	Heme-heme interaction in lamprey hemoglobin—an explanation
	W. Troll, S. Belman and E. Levine	Modification of DNA by a mutagen and carcinogens
July 16	Douglas Marsland	A pressure analysis of the effects of heavy water (D_2O) on the form, movement and plasmagel structure of *Amoeba proteus*
	R. H. Cheney	Variations in resistance to radiation of Arbacia correlated with stages in development
	C. C. Speidel	Motion picture showing comparative resistance of Arbacia zygotes to injury by gamma or ultraviolet irradiation
July 23	R. D. Allen	Polarized light microscopy by means of a birefringence detector and scanning arrangement
	A. J. de Lorenzo	Ultrastructure of a crayfish caudal photoreceptor under conditions of light and darkness; a tale of hindsight
July 30	P. Person and D. Philpott	Limulus gill cartilage: a "plant-like" animal tissue
	S. Simpson	Analysis of lizard tail regeneration: the role of the ependyma
	M. M. Rapport	Ebb-tide antigens in the brain

August 6	W. A. Siddiqui	A helical structure in ribonucleoprotein bodies of *Entamoeba invadens*
	Max A. Lauffer	1. A thermodynamic definition of hydration 2. Membrane equilibrium in multicomponent systems
	M. E. Kriebel	Physiology of the tunicate heart (film)
August 13	Ruth Hubbard and John E. Dowling	Effect of flashes on light and dark adaptation
	Clara F. Armstrong	Morphological mechanism of intracellular impulse conduction in striated muscle
	A. B. Novikoff	Lysosomes in thyroid epithelium of untreated, TSH-stimulated, and I^{131}-irradiated rats
	Hans Laufer, Yasukiyo Nakose and Jerome Vanderberg	Nucleo-cytoplasmic interactions in the development of the salivary gland of *Chironomus thummi* (Diptera)
August 20	F. N. Sudak	Dynamics and energetics of the circulatory system of dog fish
	George Szabó, T. Fitzpatrick and G. Wilgram	The pigmentary system of the squid
	F. C. G. Hoskin	Stimulation by specific quinones of glucose metabolism by brain
	F. M. Child	Flagellar regeneration: a mechanism accounting for its initiation and regulation

10. MEMBERS OF THE CORPORATION, 1963

LIFE MEMBERS

ADOLPH, DR. EDWARD F., University of Rochester, School of Medicine and Dentistry, Rochester, New York
BRODIE, MR. DONALD, 522 Fifth Avenue, New York 18, New York
COLE, DR. ELBERT C., 2 Chipman Park, Middlebury, Vermont
COWDRY, DR. E. V., 4580 Scott Avenue, St. Louis 10, Missouri
CRANE, MRS. W. MURRAY, 820 Fifth Avenue, New York 21, New York
HESS, DR. WALTER, 737 Maple Street, Spartanburg, South Carolina
HISAW, DR. F. L., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts
JACOBS, DR. M. H., Department of Physiology, University of Pennsylvania, Philadelphia 4, Pennsylvania
KNOWLTON, DR. F. P., c/o Mr. G. L. Gravelt, Jamesville, Rt. 2, New York
LEWIS, DR. W. H., Johns Hopkins University, Baltimore, Maryland
LOWTHER, DR. FLORENCE, Barnard College, New York, New York
MACDOUGALL, DR. MARY STUART, Mt. Vernon Apartments, 423 Clairmont Avenue, Decatur, Georgia
MALONE, DR. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
MEANS, DR. J. H., 15 Chestnut Street, Boston, Massachusetts
MEDES, DR. GRACE, 303 Abington Avenue, Philadelphia 11, Pennsylvania

Moore, Dr. J. Percy, RD No. 1, Box 347, Chapel Hill, North Carolina
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Plough, Dr. H. H., Amherst College, Amherst, Massachusetts
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania
Scott, Dr. Ernest L., Columbia University, New York, New York
Schrader, Dr. Sally, Duke University, Durham, North Carolina
Turner, Dr. C. L., Northwestern University, Evanston, Illinois
Waite, Dr. F. G., 144 Locust Street, Dover, New Hampshire
Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California
Warren, Dr. Herbert S., 2768 Egypt Road, Audubon, Pennsylvania
Whedon, Dr. A. D., 21 Lawncrest, Danbury, Connecticut

Regular Members

Abell, Dr. Richard G., 55 East 2nd Avenue, New York 28, New York
Adelman, Dr. William J., Department of Physiology, University of Maryland Medical School, Baltimore 1, Maryland
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota
Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania
Allen, Dr. Robert D., Department of Biology, Princeton University, Princeton, New Jersey
Alscher, Dr. Ruth, Department of Physiology, Manhattanville College, Purchase, New York
Amatniek, Dr. Ernest, 34 Horner Avenue, Hastings-on-the-Hudson, New York
Amberson, Dr. William R., Woods Hole, Massachusetts
Anderson, Dr. J. M., Department of Zoology, Cornell University, Ithaca, New York
Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland (Send mail to Box 632, Edgewood, Maryland)
Armstrong, Dr. Philip B., Department of Anatomy, State University of New York, College of Medicine, Syracuse 10, New York
Arnold, Dr. William A., Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee
Atwood, Dr. Kimball C., 702 West Pennsylvania Avenue, Urbana, Illinois.
Auclair, Dr. Walter, Department of Biological Sciences, University of Cincinnati, Cincinnati 21, Ohio
Austin, Dr. C. R., Physiological Laboratory, Downing Street, Cambridge, England
Austin, Dr. Mary L., 506½ North Indiana Avenue, Bloomington, Indiana
Ayers, Dr. John C., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Baitsell, Dr. George A., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut
Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts
Ballard, Dr. William W., Department of Zoology, Dartmouth College, Hanover, New Hampshire

Baltus, Dr. Elyane, Laboratoire de Morphologie Animale, 1850 Chaussée de Wavre, Bruxelles 16, Belgique

Bang, Dr. F. B., Department of Pathobiology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

Bard, Dr. Phillip, Johns Hopkins Medical School, Baltimore, Maryland

Barth, Dr. L. G., Department of Zoology, Columbia University, New York 27, New York

Barth, Dr. Lucena, Department of Zoology, Barnard College, New York 27, New York

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois

Baylor, Dr. E. A., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Baylor, Dr. Martha B., Marine Biological Laboratory, Woods Hole, Massachusetts

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa

Beck, Dr. L. V., Department of Pharmacology, Indiana University, School of Experimental Medicine, Bloomington, Indiana

Behre, Dr. Elinor M., Black Mountain, North Carolina

Bennett, Dr. Michael V., Department of Neurology, College of Physicians and Surgeons, New York 32, New York

Bennett, Dr. Miriam F., Department of Biology, Sweet Briar College, Sweet Briar, Virginia

Berg, Dr. William E., Department of Zoology, University of California, Berkeley 4, California

Berman, Dr. Mones, Institute for Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland

Bernheimer, Dr. Alan W., New York University College of Medicine, New York 16, New York

Bernstein, Dr. Maurice, Department of Anatomy, Wayne State University College of Medicine, Detroit 7, Michigan

Bertholf, Dr. Lloyd M., Illinois Wesleyan University, Bloomington, Illinois

Bevelander, Dr. Gerrit, Medical Center, University of Texas, Dental Branch, Houston, Texas 77025

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge 38, Massachusetts

Bishop, Dr. David W., Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore 10, Maryland

Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore 5, Maryland

Bloch, Dr. Robert, 518 South 42nd Street, Apt. C7, Philadelphia 4, Pennsylvania

Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey

Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68th Street, New York 21, New York

Bodian, Dr. David, Department of Anatomy, Johns Hopkins University, 709 North Wolfe Street, Baltimore 5, Maryland

Boell, Dr. Edgar J., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Connecticut

Bold, Dr. Harold C., Department of Botany, University of Texas, Austin, Texas

Borei, Dr. Hans G., Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

Bowen, Dr. Vaughan T., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California

Bridgman, Dr. Anna J., Department of Biology, Agnes Scott College, Decatur, Georgia

Brinley, Dr. F. J., Jr., Department of Physiology, Johns Hopkins Medical School, Baltimore 5, Maryland

Bronk, Dr. Detlev W., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Brooks, Dr. Matilda M., Department of Physiology, University of California, Berkeley 4, California

Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois

Brownell, Dr. Katherine A., Department of Physiology, Ohio State University, Columbus, Ohio

Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Maryland

Bullock, Dr. T. H., Department of Zoology, University of California, Los Angeles 24, California

Burbanck, Dr. William D., Emory University, Box 15134, Atlanta, Georgia 30333

Burdick, Dr. C. Lalor, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware

Burkenroad, Dr. M. D., 3169 Bremerton Pl., La Jolla, Calif. 92037

Butler, Dr. E. G., Department of Biology, P. O. Box 704, Princeton University, Princeton, New Jersey

Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas

Cantoni, Dr. Giullio, National Institutes of Health, Mental Health, Bethesda 14, Maryland

Carlson, Dr. Francis D., Department of Biophysics, Johns Hopkins University, Baltimore 18, Maryland

Carpenter, Dr. Russell L., Tufts University, Medford 55, Massachusetts

Carriker, Dr. Melbourne R., Marine Biological Laboratory, Woods Hole, Massachusetts

Carson, Miss Rachel, 11701 Berwick Road, Silver Spring, Maryland

Case, Dr. James, Department of Biology, University of California, Santa Barbara, Goleta, California

Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York 21, New York

Chaet, Dr. Alfred B., Department of Biology, American University, Washington 16, D. C.

Chambers, Dr. Edward, Department of Physiology, University of Miami Medical School, Coral Gables, Florida

Chang, Dr. Joseph J., Inst. f. physikal Chemie an der Techn. Hochscule, Aachen, Germany

Chase, Dr. Aurin M., Department of Biology, Princeton University, Princeton, New Jersey

Cheney, Dr. Ralph H., Biological Laboratory, Brooklyn College, Brooklyn 10, New York

Child, Dr. Frank M., Department of Zoology, University of Chicago, Chicago 37, Illinois

Claff, Dr. C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts

Clark, Dr. A. M., Department of Biological Sciences, University of Delaware, Newark, Delaware

Clark, Dr. Eloise E., Department of Zoology, Columbia University, New York 27, New York

Clark, Dr. E. R., 315 S. 41st Street, Philadelphia 4, Pennsylvania

Clark, Dr. Leonard B., Department of Biology, Union College, Schenectady, New York

Clarke, Dr. George L., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Cleland, Dr. Ralph E., Department of Botany, Indiana University, Bloomington, Indiana

Clement, Dr. A. C., Department of Biology, Emory University, Atlanta 22, Georgia

Cohen, Dr. Seymour S., Department of Chemistry, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Cole, Dr. Kenneth S., (NINDB), National Institutes of Health, Bethesda 14, Maryland

Collett, Dr. Mary E., 34 Weston Road, Wellesley 81, Massachusetts

Collier, Dr. Jack R., Marine Biological Laboratory, Woods Hole, Massachusetts

Colton, Dr. H. S., Box 699, Flagstaff, Arizona

Colwin, Dr. Arthur L., Department of Biology, Queens College, Flushing, New York

Colwin, Dr. Laura H., Department of Biology, Queens College, Flushing, New York

Cooper, Dr. Kenneth W., Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire

Cooperstein, Dr. Sherwin J., Department of Anatomy, Western Reserve University Medical School, Cleveland, Ohio

Copeland, Dr. Eugene, Zoology Department, Tulane University, New Orleans, Louisiana 70185

Copeland, Dr. Manton, Bowdoin College, Brunswick, Maine

Cornman, Dr. Ivor, Hazleton Laboratories, Box 333, Falls Church, Virginia

Costello, Dr. Donald P., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Costello, Dr. Helen Miller, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Crane, Mr. John O., Woods Hole, Massachusetts

Crane, Dr. Robert K., Department of Biochemistry, The Chicago Medical School, Chicago 12, Illinois

Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire

Crouse, Dr. Helen V., Department of Botany, Columbia University, New York 27, New York

Crowell, Dr. Sears, Department of Zoology, Indiana University, Bloomington, Indiana

Csapo, Dr. Arpad I., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Curtis, Dr. Maynie R., Box 8215, University Branch, Coral Gables 46, Florida

Curtis, Dr. W. C., 504 Westmount Avenue, Columbia, Missouri

Dan, Dr. Jean Clark, Misaki Biological Station, Misaki, Japan

Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan

Danielli, Dr. James F., Department of Medicinal Chemistry, University of Buffalo School of Pharmacy, Buffalo 14, New York

Davis, Dr. Bernard D., Harvard Medical School, 25 Shattuck Street, Boston 15, Massachusetts

Dawson, Dr. A. B., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Dawson, Dr. J. A., 129 Violet Avenue, Floral Park, Long Island, New York

Deane, Dr. Helen W., Albert Einstein College of Medicine, New York 61, New York

Dettbarn, Dr. Wolf-Dietrich, Department of Neurology, College of Physicians and Surgeons, New York 32, New York

Diller, Dr. Irene C., Institute for Cancer Research, Fox Chase, Philadelphia 11, Pennsylvania

Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania

Dodds, Dr. G. S., West Virginia University School of Medicine, Morgantown, West Virginia

Dolley, Dr. William L., Trevillans, Virginia

Doolittle, Dr. R. F., Chemistry II, Karolinska Institute, Stockholm 60, Sweden

Dowben, Dr. Robert, Biology Department, Massachusetts Institute of Technology, Cambridge, Massachusetts

Duryee, Dr. William R., George Washington University, 2300 K Street N.W., Washington, D. C.

Ebert, Dr. James David, Director, Carnegie Institute of Washington, 115 W. University Parkway, Baltimore 10, Maryland

Eckert, Dr. Roger O., Department of Zoology, Syracuse University, Syracuse 10, New York

Edds, Dr. Mac V., Jr., Department of Biology, Brown University, Providence 12, Rhode Island

Edwards, Dr. Charles, Department of Physiology, University of Minnesota, Minneapolis 14, Minnesota

Eichel, Dr. Herbert J., Department of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pennsylvania

Eisen, Dr. Herman, Department of Medicine, Washington University, St. Louis, Missouri

Elliott, Dr. Alfred M., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Essner, Dr. Edward S., Department of Pathology, Albert Einstein College of Medicine, New York 61, New York

Evans, Dr. Titus C., State University of Iowa College of Medicine, Iowa City, Iowa

Failla, Dr. P. M., Radiological Physics Division, Argonne National Laboratory, Argonne, Illinois

Farmanfarmaian, Dr. Allahverdi, Professor of General Physiology, Faculty of Medicine, Pahlavi University, Shiraz, Iran

Fauré-Fremiet, Dr. Emmanuel, Collège de France, Paris, France

Fawcett, Dr. D. W., Department of Anatomy, Harvard Medical School, Boston 15, Massachusetts

Ferguson, Dr. F. P., Division of General Medical Sciences, National Institutes of Health, Bethesda 14, Maryland

Ferguson, Dr. James K. W., Connought Laboratories, University of Toronto, Toronto, Ontario, Canada

Figge, Dr. F. H. J., University of Maryland Medical School, Lombard and Green Streets, Baltimore 1, Maryland

Fingerman, Dr. Milton, Department of Zoology, Newcomb College, Tulane University, New Orleans 18, Louisiana

Fischer, Dr. Ernst, Department of Physiology, Medical College of Virginia, Richmond, Virginia

Fisher, Dr. Frank M., Jr., Department of Biology, Rice University, Houston 1, Texas

Fisher, Dr. Jeanne M., Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

Fisher, Dr. Kenneth C., Department of Biology, University of Toronto, Toronto, Ontario, Canada

Forbes, Dr. Alexander, 16 Divinity Avenue, Cambridge, Massachusetts

Fraenkel, Dr. Gottfried S., Department of Entomology, University of Illinois, Urbana, Illinois

Freygang, Dr. Walter H., Jr., 6247 29th St. N.W., Washington 15, D. C.

Fries, Dr. Erik F. B., Box 605, Woods Hole, Masachusetts

Fuortes, Dr. Michael C. F., NINDB, National Institutes of Health, Bethesda 14, Maryland

Furshpan, Dr. Edwin J., Department of Neurophysiology, Harvard Medical School, Boston 15, Massachusetts

Furth, Dr. Jacob, 99 Fort Washington Avenue, New York 32, New York

Fye, Dr. Paul M., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Gabriel, Dr. Mordecai, Department of Biology, Brooklyn College, Brooklyn 10, New York

Gaffron, Dr. Hans, Department of Biology, Florida State University, Institute of Molecular Biophysics, Tallahassee, Florida

Gall, Dr. Joseph G., Department of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Galtsoff, Dr. Paul S., Woods Hole, Massachusetts

Gilbert, Dr. Daniel L., National Institutes of Health, Laboratory of Biophysics, NINDB, Bethesda 14, Maryland

Gilman, Dr. Lauren C., Department of Zoology, University of Miami, Coral Gables, Florida

Ginsberg, Dr. Harold S., Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

Goldsmith, Dr. Timothy H., Department of Zoology, Yale University, New Haven, Connecticut

Goldstein, Dr. Lester, Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

Goodchild, Dr. Chauncey G., Department of Biology, Emory University, Atlanta 22, Georgia

Goodrich, Dr. H. B., Department of Biology, Wesleyan University, Middletown, Connecticut

Gotschall, Dr. Gertrude Y., 315 East 68th Street, Apt. 9M, New York, New York 10021

Graham, Dr. Herbert, U. S. Fish and Wildlife Service, Woods Hole, Massachusetts

Grand, Mr. C. G., Cancer Institute of Miami, 1155 N. W. 15th Street, Miami, Florida

Grant, Dr. Philip, National Science Foundation, 1951 Constitution Avenue, Washington 25, D. C.

Gray, Dr. Irving E., Department of Zoology, Duke University, Durham, North Carolina

Green, Dr. James W., Department of Physiology, Rutgers University, New Brunswick, New Jersey

Green, Dr. Maurice, Department of Microbiology, St. Louis University Medical School, St. Louis, Missouri

Gregg, Dr. James H., Department of Biological Sciences, University of Florida, Gainesville, Florida

Gregg, Dr. John R., Department of Zoology, Duke University, Durham, North Carolina

Greif, Dr. Roger L., Department of Physiology, Cornell University Medical College, New York 21, New York

Griffin, Dr. Donald F., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Grosch, Dr. Daniel S., Department of Genetics, Gardner Hall, North Carolina State College, Raleigh, North Carolina

Gross, Dr. Paul, Department of Biology, Brown University, Providence 12, Rhode Island

Grundfest, Dr. Harry, College of Physicians and Surgeons, Columbia University, New York 32, New York

Guttman, Dr. Rita, Department of Physiology, Brooklyn College, Brooklyn 10, New York

Gwilliam, Dr. G. F., Department of Biology, Reed College, Portland 2, Oregon

Hajdu, Dr. Stephen, National Institutes of Health, Bethesda 14, Maryland

Hall, Dr. Frank G., Department of Physiology, Duke University Medical School, Durham, North Carolina

Hamburger, Dr. Viktor, Department of Zoology, Washington University, St. Louis, Missouri

Hamilton, Dr. Howard L., Department of Biology, University of Virginia, Charlottesville, Va.

Hance, Dr. Robert T., RR #3, 6609 Smith Road, Loveland, Ohio

Harding, Dr. Clifford V., Jr., Columbia University, College of Physicians & Surgeons, 630 W. 168th St., New York, New York 10032

Harnley, Dr. Morris H., Washington Square College, New York University, New York 3, New York

Hartline, Dr. H. Keffer, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Hartman, Dr. Frank A., Ohio State University, Hamilton Hall, Columbus, Ohio

Hartman, Dr. P. E., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Harvey, Dr. Ethel Browne, Marine Biological Laboratory, Woods Hole, Massachusetts

Hastings, Dr. J. Woodland, Division of Biochemistry, University of Illinois, Urbana, Illinois

Hauschka, Dr. T. S., Roswell Park Memorial Institute, 666 Elm Street, Buffalo 3, New York

Haxo, Dr. Francis T., Division of Marine Botany, Scripps Institution of Oceanography, University of California, La Jolla, California

Hayashi, Dr. Teru, Department of Zoology, Columbia University, New York 27, New York

Hayden, Dr. Margaret A., 34 Weston Road, Wellesley 81, Massachusetts

Haywood, Dr. Charlotte, P. O. Box 14, South Hadley, Massachusetts

Hendley, Dr. Charles D., 615 South Avenue, Highland Park, New Jersey

Henley, Dr. Catherine, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Herndon, Dr. Walter R., Botany Department, University of Tennessee, Knoxville, Tennessee

Hervey, Mr. John P., Box 735, Woods Hole, Massachusetts

Hessler, Dr. Anita Y., Marine Biological Laboratory, Woods Hole, Massachusetts

Hiatt, Dr. Howard H., Department of Medicine, Harvard Medical School, Boston 15, Massachusetts

Hibbard, Dr. Hope, Department of Zoology, Oberlin College, Oberlin, Ohio

Hill, Dr. Samuel E., 135 Brunswick Road, Troy, New York

Hirshfield, Dr. Henry I., Department of Biology, New York University, Washington Square College, New York 3, New York

Hoadley, Dr. Leigh, Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Hodes, Dr. Robert, Department of Pediatrics, Mount Sinai Hospital, New York 39, New York

HODGE, DR. CHARLES, IV, Department of Biology, Temple University, Philadelphia 22, Pennsylvania

HOFFMAN, DR. JOSEPH, National Heart Institute, National Institutes of Health, Bethesda 14, Maryland

HOLLAENDER, DR. ALEXANDER, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

HOLZ, DR. GEORGE G., JR., Dept. of Microbiology, S. U. N. Y., Upstate Medical Center, Syracuse, New York

HOPKINS, DR. HOYT S., 59 Heatherdell Road, Ardsley, New York

HOSKIN, DR. FRANCIS C. G., Department of Neurology, College of Physicians & Surgeons, New York 32, New York

HUNTER, DR., FRANCIS R., University of the Andes, Calle 18-a Carrera I-E, Bogota, Colombia, South America

HUNTER, DR. W. D. R., Department of Zoology, Lyman Hall, Syracuse University, Syracuse 10, New York

HURWITZ, DR. J., Department of Molecular Biology, Albert Einstein College of Medicine, Eastchester Road and Morris Park Ave., Bronx, New York 10461

HUTCHENS, DR. JOHN E., Department of Physiology, University of Chicago, Chicago 37, Illinois

HYDE, DR. BEAL B., Department of Plant Sciences, University of Oklahoma, Norman, Oklahoma

HYMAN, DR. LIBBIE H., American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

INOUÉ, DR. S., Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire

IRVING, DR. LAURENCE, Laboratory of Zoophysiology, University of Alaska, College, Alaska

ISENBERG, DR. IRVIN, Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Massachusetts

ISELIN, MR. COLUMBUS O'D., Woods Hole, Massachusetts

ISSELBACKER, DR. KURT J., Massachusetts General Hospital, Boston 14, Massachusetts

JENNER, DR. CHARLES E., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

JOHNSON, DR. FRANK H., Department of Biology, Princeton University, Princeton, New Jersey

JONES, DR. E. RUFFIN, JR., Department of Biological Sciences, University of Florida, Gainesville, Florida

JONES, DR. RAYMOND F., Department of Biology, Princeton University, Princeton, New Jersey

JOSEPHSON, DR. R. K., Department of Zoology, University of Minnesota, Minneapolis 14, Minnesota

KAAN, DR. HELEN W., Marine Biological Laboratory, Woods Hole, Massachusetts

KABAT, DR. E. A., Neurological Institute, College of Physicians and Surgeons, New York 32, New York

KAMINER, DR. B., Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Massachusetts

Kane, Dr. Robert E., Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire

Karush, Dr. Fred, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

Kaufman, Dr. B. P., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Kemp, Dr. Norman E., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Kempton, Dr. Rudolf T., Department of Zoology, Vassar College, Poughkeepsie, New York

Keosian, Dr. John, Department of Biology, Rutgers University, Newark 2, New Jersey

Ketchum, Dr. Bostwick H., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Keynan, Dr. Alexander, Institute for Biological Research, Ness-Ziona, Israel

Kille, Dr. Frank R., State Department of Education, Albany 1, New York

Kind, Dr. C. Albert, Department of Zoology, University of Connecticut, Storrs, Connecticut

Kindred, Dr. J. E., University of Virginia, Charlottesville, Virginia

King, Dr. Robert L., State University of Iowa, Iowa City, Iowa

Kingsbury, Dr. John M., Department of Botany, Cornell University, Ithaca, New York

Kisch, Dr. Bruno, 71 Maple Street, Brooklyn 25, New York

Klein, Dr. Morton, Department of Microbiology, Temple University, Philadelphia, Pennsylvania

Kleinholz, Dr. Lewis H., Department of Biology, Reed College, Portland 2, Oregon

Klotz, Dr. I. M., Department of Chemistry, Northwestern University, Evanston, Illinois

Kolin, Dr. Alexander, Department of Biophysics, California Medical School, Los Angeles 24, California

Korr, Dr. I. M., Department of Physiology, Kirksville College of Osteopathy, Kirksville, Missouri

Krahl, Dr. M. E., Department of Physiology, University of Chicago, Chicago 37, Illinois

Krane, Dr. Stephen M., Massachusetts General Hospital, Boston 14, Massachusetts

Krauss, Dr. Robert, Department of Botany, University of Maryland, Baltimore, Maryland

Kreig, Dr. Wendell J. S., 303 East Chicago Avenue, Chicago, Illinois

Kuffler, Dr. Stephen W., Department of Pharmacology, Harvard Medical School, Boston 15, Massachusetts

Kunitz, Dr. Moses, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Lamy, Dr. Francois, Department of Anatomy, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

Lancefield, Dr. D. E., Queens College, Flushing, New York

LANCEFIELD, DR. REBECCA C., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

LANDIS, DR. E. M., Harvard Medical School, Boston 15, Massachusetts

LANSING, DR. ALBERT I., Department of Anatomy, University of Pittsburgh Medical School, Pittsburgh 13, Pennsylvania

LASH, DR. JAMES W., Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

LAUFER, DR. H., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

LAUFFER, DR. MAX A., Department of Biophysics, University of Pittsburgh, Pittsburgh 13, Pennsylvania

LAWLER, DR. H. CLAIRE, Department of Biochemistry and Neurology, College of Physicians and Surgeons, New York 32, New York

LAVIN, DR. GEORGE I., 6200 Norvo Road, Baltimore 7, Maryland

LAZAROW, DR. ARNOLD, Department of Anatomy, University of Minnesota Medical School, Minneapolis 14, Minnesota

LEDERBERG, DR. JOSHUA, Department of Genetics, Stanford Medical School, Palo Alto, California

LEE, DR. RICHARD E., Cornell University College of Medicine, New York 21, New York

LEFEVRE, DR. PAUL G., University of Louisville School of Medicine, Louisville, Kentucky

LEHMANN, DR. FRITZ, Zoologische Institut, University of Berne, Berne, Switzerland

LEVINE, DR. RACHMIEL, New York Medical College, Department of Medicine, 5th Avenue at 106th Street, New York 29, New York

LEVY, DR. MILTON, Department of Biochemistry, New York University School of Dentistry, New York 10, New York

LEWIN, DR. RALPH A., Scripps Institution of Oceanography, La Jolla, California

LEWIS, DR. HERMAN W., Genetic Biology Program, National Science Foundation, Washington 25, D. C.

LEWIS, DR. IVEY F., 800 Rugby Road, Charlottesville, Virginia

LING, DR. GILBERT, 307 Berkeley Road, Merion, Pennsylvania

LITTLE, DR. E. P., 216 Highland Street, West Newton, Massachusetts

LLOYD, DR. DAVID P. C., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

LOCHHEAD, DR. JOHN H., Department of Zoology, University of Vermont, Burlington, Vermont

LOEB, DR. R. F., 950 Park Avenue, New York 28, New York

LOEWENSTEIN, DR. WERNER R., Department of Physiology, College of Physicians and Surgeons, New York 32, New York

LOFTFIELD, DR. ROBERT B., Massachusetts General Hospital, Boston, Massachusetts

LORAND, DR. LASZLO, Department of Chemistry, Northwestern University, Evanston, Illinois

DE LORENZO, DR. ANTHONY, Anatomical and Pathological Research Laboratories, Johns Hopkins Hospital, Baltimore 5, Maryland

Love, Dr. Warner E., 1043 Marlau Drive, Baltimore 12, Maryland
Lubin, Dr. Martin, Department of Pharmacology, Harvard Medical School, Boston 15, Massachusetts
Lynch, Dr. Clara J., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York
Lynn, Dr. W. Gardner, Department of Biology, Catholic University of America, Washington 17, D. C.
McCann, Dr. Frances, Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire
McCouch, Dr. Margaret Sumwalt, University of Pennsylvania Medical School, Philadelphia 4, Pennsylvania
McDonald, Sister Elizabeth Seton, Department of Biology, College of Mt. St. Joseph, Mt. St. Joseph, Ohio
McDonald, Dr. Margaret R., Waldermar Medical Research Foundation, Sunnyside Blvd. and Waldermar Road, Woodbury R.D., New York
McElroy, Dr. William D., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland
Maas, Dr. Werner K., New York University College of Medicine, New York City, New York
Magruder, Dr. Samuel R., Department of Anatomy, Tufts Medical School, 135 Harrison Avenue, Boston, Massachusetts
Manwell, Dr. Reginald D., Department of Zoology, Syracuse University, Syracuse 10, New York
Marks, Dr. Paul A., Columbia University, College of Physicians and Surgeons, New York 32, New York
Marshak, Dr. Alfred, Department of Radiology, Jefferson Medical College, Philadelphia 7, Pennsylvania
Marsland, Dr. Douglas A., 48 Church Street, Woods Hole, Massachusetts
Martin, Dr. Earl A., 682 Rudder Road, Naples, Florida 33940
Mathews, Dr. Samuel A., Thompson Biological Laboratory, Williams College, Williamstown, Massachusetts
Mazia, Dr. Daniel, Department of Zoology, University of California, Berkeley 4, California
Meinkoth, Dr. Norman, Department of Biology, Swarthmore College, Swarthmore, Pennsylvania
Metz, Dr. C. B., Institute for Space Biosciences, Florida State University, Tallahassee, Florida
Metz, Dr. Charles W., Box 714, Woods Hole, Massachusetts
Middlebrook, Dr. Robert, Dartmouth Medical Center, Hanover, New Hampshire
Milkman, Dr. Roger D., Department of Zoology, Syracuse University, Syracuse 10, New York
Miller, Dr. J. A., Jr., Department of Anatomy, Tulane University Medical School, New Orleans 18, Louisiana
Milne, Dr. Lorus J., Department of Zoology, University of New Hampshire, Durham, New Hampshire
Moe, Mr. Henry A., Guggenheim Memorial Foundation, 551 Fifth Avenue, New York 17, New York

Monroy, Dr. Alberto, Institute of Comparative Anatomy, University of Palermo, Italy

Moore, Dr. George M., Department of Zoology, University of New Hampshire, Durham, New Hampshire

Moore, Dr. John A., Department of Zoology, Columbia University, New York 27, New York

Moore, Dr. John W., Department of Physiology, Duke University Medical Center, Durham, North Carolina

Moore, Dr. R. O., Department of Biochemistry, Ohio State University, Columbus 10, Ohio

Moran, Dr. Joseph, Department of Biology, Russell Sage College, Troy, New York

Morrill, Dr. John B., Jr., Department of Biology, Wesleyan University, Middletown, Connecticut

Moscona, Dr. A. A., Department of Zoology, University of Chicago, Chicago 37, Illinois

Moul, Dr. E. T., Department of Botany, Rutgers University, New Brunswick, New Jersey

Mountain, Mrs. J. D., Charles Road, Mt. Kisco, New York

Mullins, Dr. Lorin J., Department of Biophysics, University of Maryland School of Medicine, Baltimore 1, Maryland

Musacchia, Dr. Xavier, Department of Biology, St. Louis University, St. Louis 4, Missouri

Nabrit, Dr. S. M., President, Texas Southern University, 3201 Wheeler Avenue, Houston 4, Texas

Nace, Dr. Paul Foley, Department of Biology, Hamilton College, McMaster University, Hamilton, Ontario, Canada

Nachmansohn, Dr. David, Columbia University, College of Physicians and Surgeons, New York 32, New York

Nasatir, Dr. Maimon, Department of Botany, Brown University, Providence, Rhode Island

Navez, Dr. Albert E., 206 Churchill's Lane, Milton 86, Massachusetts

Nelson, Dr. Leonard, Department of Physiology, Emory University, Atlanta 22, Georgia

Nason, Dr. Alvin, McCollum-Pratt Institute, The Johns Hopkins University, Baltimore 18, Maryland

Neurath, Dr. H., Department of Biochemistry, University of Washington, Seattle 5, Washington

Nicoll, Dr. Paul A., Block Oak Lodge, RR #2, Bloomington, Indiana

Niu, Dr. Man-Chiang, Temple University, Philadelphia 22, Pennsylvania

Novikoff, Dr. Alex B., Department of Pathology, Albert Einstein College of Medicine, New York 61, New York

Ochoa, Dr. Severo, New York University College of Medicine, New York 16, New York

Odum, Dr. Eugene, Department of Zoology, University of Georgia, Athens, Georgia

Oppenheimer, Dr. Jane M., Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania

Osterhout, Dr. W. J. V., 450 East 63rd Street, New York 21, New York

Osterhout, Dr. Marion Irwin, 450 East 63rd St., New York 21, New York

Packard, Dr. Charles, Woods Hole, Massachusetts

Page, Dr. Irvine H., Cleveland Clinic, Cleveland, Ohio

Palmer, Dr. John D., Department of Biology, University of Illinois, Navy Pier, Chicago 11, Illinois

Parpart, Dr. Arthur K., Department of Biology, Princeton University, Princeton, New Jersey

Passano, Dr. Leonard M., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Patten, Dr. Bradley M., University of Michigan School of Medicine, Ann Arbor, Michigan

Perkins, Dr. John F., Jr., Department of Physiology, University of Chicago, Chicago 37, Illinois

Person, Dr. Philip, Chief, Special Dental Research Program, VA Hospital, Brooklyn 9, New York

Pettibone, Dr. Marian H., U. S. National Museum, Division of Marine Interbebrates, Washington 25, D. C.

Philpott, Dr. Delbert E., Department of Biochemistry, University of Colorado Medical Center, 4200 East Ninth Ave., Denver 20, Colorado

Pick, Dr. Joseph, Department of Anatomy, New York University, Bellevue Medical Center, New York 16, New York

Pierce, Dr. Madelene E., Department of Zoology, Vassar College, Poughkeepsie, New York

Pollister, Dr. A. W., Department of Zoology, Columbia University, New York 27, New York

Pond, Dr. Samuel E., 53 Alexander Street, Manchester, Connecticut

Porter, Dr. Keith R., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Potter, Dr. David, Department of Neurophysiology, Harvard Medical School, Boston 15, Massachusetts

Proctor, Dr. Nathaniel, Department of Biology, Morgan State College, Baltimore 12, Maryland

Prosser, Dr. C. Ladd, Department of Physiology, Burrill Hall, University of Illinois, Urbana, Illinois

Provasoli, Dr. Luigi, Haskins Laboratories, 305 E. 43rd Street, New York 17, New York

Ramsey, Dr. Robert W., Medical College of Virginia, Richmond, Virginia

Rankin, Dr. John S., Department of Zoology, University of Connecticut, Storrs, Connecticut

Ranzi, Dr. Silvio, Department of Zoology, University of Milan, Milan, Italy

Rapport, Dr. M., Department of Biochemistry, Albert Einstein College of Medicine, New York 61, New York

Ratner, Dr. Sarah, Public Health Research Institute of the City of New York, Foot of East 15th Street, New York 9, New York

Ray, Dr. Charles, Jr., Department of Biology, Emory University, Atlanta 22, Georgia

Read, Dr. Clark P., Department of Biology, Rice University, Houston, Texas

Rebhun, Dr. Lionel I., Department of Biology, Box 704, Princeton University, Princeton, New Jersey

Recknagel, Dr. R. O., Department of Physiology, Western Reserve University, Cleveland, Ohio

Redfield, Dr. Alfred C., Woods Hole, Massachusetts

Renn, Dr. Charles E., 509 Ames Hall, Johns Hopkins University, Baltimore 18, Maryland

Reuben, Dr. John P., Department of Neurology, College of Physicians and Surgeons, New York 32, New York

Reznikoff, Dr. Paul, Cornell University Medical College, 1300 York Avenue, New York 16, New York

Rich, Dr. Alexander, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

Richards, Dr. A., 2950 E. Mabel Street, Tucson, Arizona

Richards, Dr. A. Glenn, Department of Entomology, University of Minnesota, St. Paul 1, Minnesota

Richards, Dr. Oscar W., American Optical Company Research Center, Southbridge, Massachusetts

Rockstein, Dr. Morris, Medical Research Building, 1600 N. W. 10th Avenue, Miami, Florida

Rogick, Dr. Mary D., College of New Rochelle, New Rochelle, New York

Romer, Dr. Alfred S., Harvard University, Museum of Comparative Zoology, Cambridge 38, Massachusetts

Ronkin, Dr. Raphael R., National Science Foundation Course Content Improvement Section, Washington 25, D. C.

Root, Dr. R. W., Department of Biology, College of the City of New York, New York City, New York

Root, Dr. W. S., Department of Physiology, Columbia University College of Physicians and Surgeons, New York 32, New York

Rose, Dr. S. Meryl, Department of Anatomy, Tulane University, New Orleans 18, Louisiana

Rosenberg, Dr. Evelyn K., Department of Pathology, New York University Bellevue Medical Center, New York 16, New York

Rosenberg, Dr. Philip, Department of Neurology, Columbia University, New York City, New York

Rosenbluth, Miss Raja, Department of Zoology, Columbia University, New York 27, New York

Rosenthal, Dr. Theodore B., Department of Anatomy, University of Pittsburgh Medical School, Pittsburgh 13, Pennsylvania

Roslansky, Dr. John, Department of Zoology, University of Illinois, Urbana, Illinois

Roth, Dr. Jay S., Department of Zoology and Entomology, University of Connecticut, Storrs, Connecticut

Rothenberg, Dr. M. A., Scientific Director, Dugway Proving Ground, Dugway, Utah

RUGH, DR. ROBERTS, Radiological Research Laboratory, College of Physicians and Surgeons, New York 32, New York

RUNNSTRÖM, DR. JOHN, Wenner-Gren Institute, Stockholm, Sweden

RUSTAD, DR. RONALD C., Department of Radiology, Western Reserve University, Cleveland, Ohio 44106

RUTMAN, DR. ROBERT J., General Laboratory Building, 215 S. 34th Street, Philadelphia, Pennsylvania

RYTHER, DR. JOHN H., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

SAGER, DR. R., Department of Zoology, Columbia University, New York 27, New York

SANBORN, DR. RICHARD C., Department of Biological Sciences, Purdue University, Lafayette, Indiana

SANDERS, DR. HOWARD L., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

SAUNDERS, DR. JOHN, Department of Biology, Marquette University, Milwaukee 3, Wisconsin

SAUNDERS, MR. LAWRENCE, West Washington Square, Philadelphia 5, Pennsylvania

SAZ, DR. ARTHUR KENNETH, National Institutes of Health, Bethesda 14, Maryland

SCHACHMAN, DR. HOWARD K., Department of Biochemistry, University of California, Berkeley 4, California

SCHARRER, DR. ERNST, Department of Anatomy, Albert Einstein College of Medicine, New York 61, New York

SCHLESINGER, DR. R. WALTER, Department of Microbiology, Rutgers Medical School, New Brunswick, New Jersey

SCHMIDT, DR. L. H., Christ Hospital, Cincinnati, Ohio

SCHMITT, DR. FRANCIS O., Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

SCHMITT, DR. O. H., Department of Physics, University of Minnesota, Minneapolis 14, Minnesota

SCHNEIDERMAN, DR. HOWARD A., Department of Biology, Western Reserve University, Cleveland, Ohio

SCHOLANDER, DR. P. F., Scripps Institution of Oceanography, La Jolla, California

SCHOTTÉ, DR. OSCAR E., Department of Biology, Amherst College, Amherst, Massachusetts

SCHRAMM, DR. J. R., Department of Botany, Indiana University, Bloomington, Indiana

SCOTT, DR. ALLAN C., Colby College, Waterville, Maine

SCOTT, DR. D. B. MCNAIR, 15 General Laboratory Bldg., University of Pennsylvania, Philadelphia 4, Pennsylvania

SCOTT, SISTER FLORENCE MARIE, Seton Hill College, Greensburg, Pennsylvania

SCOTT, DR. GEORGE I., Department of Zoology, Oberlin College, Oberlin, Ohio

SEARS, DR. MARY, Glendon Road, Woods Hole, Massachusetts

SELIGER, DR. HOWARD H., McCollum-Pratt Institute, Johns Hopkins University, Baltimore 18, Maryland

SENFT, DR. ALFRED W., Woods Hole, Massachusetts

SEVERINGHAUS, DR. AURA E., 375 W. 250 Street, New York 71, New York

Shanes, Dr. Abraham, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

Shapiro, Dr. Herbert, 6025 N. 13th Street, Philadelphia 41, Pennsylvania

Shaver, Dr. John R., Department of Zoology, Michigan State University, East Lansing, Michigan

Shedlovsky, Dr. Theodore, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Shemin, Dr. David, Department of Biochemistry, Columbia University, New York City, New York

Sherman, Dr. I. W., Division of Life Sciences, University of California, Riverside, California

Sichel, Dr. Ferdinand J. M., University of Vermont, Burlington, Vermont

Sichel, Mrs. F. J. M., Department of Biology, Trinity College, Burlington, Vermont

Silva, Dr. Paul, Department of Botany, University of California, Berkeley 4, California

Simmons, Dr. John E., Jr., Department of Biology, Rice University, Houston 1, Texas

Slifer, Dr. Eleanor H., 308 Linsmore Avenue, Glenside, Pennsylvania

Smelser, Dr. George K., Department of Anatomy, Columbia University, New York 32, New York

Smith, Dr. Dietrich C., Department of Physiology, University of Maryland School of Medicine, Baltimore 1, Maryland

Smith, Mr. Homer P., General Manager, Marine Biological Laboratory, Woods Hole, Massachusetts

Smith, Mr. Paul Ferris, Woods Hole, Massachusetts

Smith, Dr. Ralph I., Department of Zoology, University of California, Berkeley 4, California

Sonneborn, Dr. T. M., Department of Zoology, Indiana University, Bloomington, Indiana

Sonnenblick, Dr. B. P., Rutgers University, 40 Rector Street, Newark 2, New Jersey

Spector, Dr. A., Howe Laboratories, Harvard Medical School, Boston 15, Massachusetts

Speidel, Dr. Carl C., Department of Anatomy, University of Virginia Medical School, Charlottesville, Virginia

Spiegel, Dr. Melvin, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire

Spratt, Dr. Nelson T., Department of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Spyropoulos, Dr. C. S., Bldg. 9-Rm. 140, National Institutes of Health, Bethesda 14, Maryland

Starr, Dr. Richard C., Department of Botany, Indiana University, Bloomington, Indiana

Steinbach, Dr. H. Burr, Department of Zoology, University of Chicago, Chicago 37, Illinois

Steinberg, Dr. Malcolm S., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

STEINHARDT, DR. JACINTO, Georgetown University, Washington 7, D. C.

STEPHENS, DR. GROVER C., Department of Zoology, University of Minnesota, Minneapolis 14, Minnesota

STETTEN, DR. DEWITT, Dean, Rutgers University Medical School, New Brunswick, New Jersey

STETTEN, DR. MARJORIE R., Rutgers University Medical School, New Brunswick, New Jersey

STEWART, DR. DOROTHY, Rockford College, Rockford, Illinois

STOKEY, DR. ALMA G., Department of Botany, Mount Holyoke College, South Hadley, Massachusetts

STONE, DR. WILLIAM, JR., Ophthalmic Plastics Laboratory, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts

STRAUS, DR. W. L., JR., Department of Anatomy, Johns Hopkins University Medical School, Baltimore 5, Maryland

STREHLER, DR. BERNARD L., 4115 Westview Road, Baltimore 18, Maryland

STRITTMATTER, DR. PHILIPP, Department of Biological Chemistry, Washington University Medical School, St. Louis, Missouri

STUNKARD, DR. HORACE W., American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

STURTEVANT, DR. ALFRED H., California Institute of Technology, Pasadena, California

SUDAK, DR. FREDERICK N., Department of Physiology, Albert Einstein College of Medicine, New York 61, New York

SULKIN, DR. S. EDWARD, Department of Bacteriology, University of Texas, Southwestern Medical School, Dallas, Texas

SWOPE, MR. GERARD, JR., 570 Lexington Avenue, New York 22, New York

SZABÓ, DR. GEORGE, Department of Dermatology, Massachusetts General Hospital, Boston 14, Massachusetts

SZENT GYÖRGYI, DR. ALBERT, Institute for Muscle Research, Marine Biological Laboratory, Woods Hole, Massachusetts

SZENT GYÖRGYI, DR. ANDREW G., Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire

TASAKI, DR. ICHIJI, Laboratory of Neurobiology, NIMH, National Institutes of Health, Bethesda 14, Maryland

TAYLOR, DR. ROBERT E., Laboratory of Neurophysiology, NINDB, Bethesda 14, Maryland

TAYLOR, DR. WILLIAM RANDOLPH, Department of Botany, University of Michigan, Ann Arbor, Michigan

TAYLOR, DR. W. ROWLAND, Department of Oceanography, Johns Hopkins University, Baltimore 18, Maryland

TEWINKEL, DR. LOIS E., Department of Zoology, Smith College, Northampton, Massachusetts

TOBIAS, DR. JULIAN, Department of Physiology, University of Chicago, Chicago 37, Illinois

TRACY, DR. HENRY C., 3595 Mynders #3, Memphis 11, Tennessee

TRAGER, DR. WILLIAM, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Travis, Dr. D. M., Department of Pharmacology, University of Florida, Gainesville, Florida

Trinkaus, Dr. J. Philip, Department of Zoology, Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Troll, Dr. Walter, Department of Industrial Medicine, New York University College of Medicine, New York 16, New York

Tweedell, Dr. Kenyon S., Department of Biology, University of Notre Dame, Notre Dame, Indiana

Tyler, Dr. Albert, Division of Biology, California Institute of Technology, Pasadena, California

Uretz, Dr. Robert B., Department of Biophysics, University of Chicago, Chicago 37, Illinois

de Villafranca, Dr. George W., Department of Zoology, Smith College, Northampton, Massachusetts

Villee, Dr. Claude A., Department of Biological Chemistry, Harvard Medical School, Boston 15, Massachusetts

Vincent, Dr. Walter S., Department of Anatomy, University of Pittsburgh, Pittsburgh 13, Pennsylvania

Wainio, Dr. W. W., Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

Wald, Dr. George, Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Warner, Dr. Robert C., Department of Chemistry, New York University College of Medicine, New York 16, New York

Warren, Dr. L., c/o Dr. A. Bussard, Institut Pasteur, 25 Rue de Docteur Roux, Paris, France

Waterman, Dr. T. H., Department of Zoology, 272 Gibbs Research Laboratory, Yale University, New Haven, Connecticut

Webb, Dr. Marguerite, Department of Physiology, and Bacteriology, Goucher College, Towson, Baltimore, Maryland

Weiss, Dr. Leon P., Department of Anatomy, The Johns Hopkins University School of Medicine, Baltimore 5, Maryland

Weiss, Dr. Paul A., Laboratory of Developmental Biology, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Wenrich, Dr. D. H., Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

Werman, Dr. Robert, Institute of Psychiatric Research, Indiana University Medical Center, 1100 W. Michigan Street, Indianapolis 7, Indiana

Whitaker, Dr. Douglas M., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

White, Dr. E. Grace, 1312 Edgar Avenue, Chambersburg, Pennsylvania

Whiting, Dr. Anna R., 535 West Vanderbilt Drive, Oak Ridge, Tennessee

Whiting, Dr. Phineas, 535 West Vanderbilt Drive, Oak Ridge, Tennessee

Wickersham, Mr. James H., 791 Park Avenue, New York 21, New York

Wichterman, Dr. Ralph, Department of Biology, Temple University, Philadelphia 22, Pennsylvania

Wieman, Dr. H. L., Box 485, Falmouth, Massachusetts

Wiercinski, Dr. Floyd J., 600 W. Franklin, Apt. 208, Minneapolis, Minnesota 55405

Wigley, Dr. Roland L., U. S. Fish and Wildlife Service, Woods Hole, Massachusetts

Wilber, Dr. C. G., Marine Laboratories, University of Delaware, Newark, Delaware

Willier, Dr. B. H., Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, Dr. J. Walter, Department of Biology, Brown University, Providence 12, Rhode Island

Wilson, Dr. T. Hastings, Department of Physiology, Harvard Medical School, Boston 15, Massachusetts

Wilson, Dr. Walter L., Department of Physiology, University of Vermont College of Medicine, Burlington, Vermont

Witschi, Dr. Emil, Universität Basel, Anatomisches Institut, Pestalozzistrasse 20, Basel, Switzerland

Wittenberg, Dr. Jonathan B., Department of Physiology and Biochemistry, Albert Einstein College of Medicine, New York 61, New York

Wright, Dr. Paul A., Spaulding Bldg., Department of Zoology, University of New Hampshire, Durham, New Hampshire

Wrinch, Dr. Dorothy, Department of Physics, Smith College, Northampton, Massachusetts

Yntema, Dr. C. L., Department of Anatomy, State University of New York College of Medicine, Syracuse 10, New York

Young, Dr. D. B., Main Street, North Hanover, Massachusetts

Zimmerman, Dr. A. M., Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn 3, New York

Zinn, Dr. Donald J., Department of Zoology, University of Rhode Island, Kingston, Rhode Island

Zirkle, Dr. Raymond E., Department of Radiobiology, University of Chicago, Chicago 37, Illinois

Zorzoli, Dr. Anita, Department of Physiology, Vassar College, Poughkeepsie, New York

Zullo, Dr. Victor A., Marine Biological Laboratory, Woods Hole, Massachusetts

Zweifach, Dr. Benjamin, New York University Bellevue Medical Center, New York 16, New York

Zwilling, Dr. Edgar, Department of Biology, Brandeis University, Waltham 54, Massachusetts

Associate Members

Aldrich, Miss Amey Owen
Alton, Mrs. Benjamin
Andres, Mrs. William
Armstrong, Mrs. Philip B.
Bacon, Mr. and Mrs. Robert
Ball, Mrs. Eric G.
Bakalar, Mr. and Mrs. David
Barbour, Mr. and Mrs. Lucius H.
Barrows, Mrs. Albert
Bartow, Mr. and Mrs. Clarence W.
Bartow, Mrs. Francis D.
Bartow, Mrs. Philip K.
Bartow, Mr. S. R.
Beale, Mr. and Mrs. Edward

Bell, Mrs. Arthur W.
Bigelow, Mrs. Robert Payne
Bradley, Mrs. Albert L.
Bradley, Dr. and Mrs. Charles
Brown, Mrs. Thornton
Burdick, Dr. C. Lalor
Burlingame, Mrs. F. A.
Butler, Dr. and Mrs. E. G.
Cahoon, Mrs. Samuel T.
Calkins, Mrs. Gary N.
Calkins, Mr. and Mrs. G. Nathan, Jr.
Carlton, Mr. Winslow G.
Carey, Miss Cornelia
Clark, Mrs. James B.
Claff, Mrs. C. Lloyd
Claff, Mr. Mark M.
Clark, Mrs. Leroy
Clark, Edna A.
Clark, Mr. and Mrs. W. Van Alan
Clowes, Mr. Allen W.
Clowes, Mrs. G. H. A.
Clowes, Dr. and Mrs. George H. A., Jr.
Colton, Mrs. H. Seymour
Conklin, Miss Isabel
Cramer, Mr. and Mrs. I. D. W.
Crane, Mr. John
Crane, Miss Louise
Crane, Mrs. W. Carey
Crane, Mrs. W. Murray
Crowell, Mr. and Mrs. Prince S.
Crossley, Miss Dorothy
Curtis, Dr. and Mrs. William D.
Daniels, Mr. and Mrs. Bruce
Daniels, Mr. and Mrs. F. H.
Day, Mr. and Mrs. Pomeroy
Draper, Mrs. Mary C.
Dreyer, Mr. and Mrs. F. A.
DuBois, Dr. Arthur B.
Elsmith, Mrs. Dorothy
Ewing, Mr. William
Ferguson, Mrs. James J.
Fisher, Mrs. B. C.
Forbes, Dr. Alexander
Francis, Mr. Lewis H., Jr.
Frost, Mrs. Frank J.
Gaiser, Dr. and Mrs. David W.
Galtsoff, Mrs. Eugenia
Garfield, Miss Eleanor
Garlock, Mr. Robert
Gifford, Mr. and Mrs. John A.
Gifford, Mr. and Mrs. Prosser
Gilchrist, Mr. and Mrs. John M.
Gildea, Dr. and Mrs. E. F.
Gillette, Mr. and Mrs. Robert S.
Glazebrook, Mrs. James
Green, Miss Gladys M.
Greer, Mr. and Mrs. William Hershey, Jr.
Gulesian, Mrs. Paul J.
Hamlen, Mr. and Mrs. J. Monroe
Hanna, Mr. and Mrs. Thomas C.
Harrington, Mr. and Mrs. Robert D.
Harvey, Dr. and Mrs. Richard
Hervey, Mrs. John P.
Hirschfeld, Mrs. Nathan B.
Hopkins, Mrs. Ralph H.
Houston, Mr. and Mrs. Howard E.
Janney, Mrs. Mary D.
Jewett, Mr. and Mrs. G. F., Jr.
Jones, Mr. and Mrs. DeWitt C., Jr.
Keith, Mrs. Harold C.
Koller, Dr. and Mrs. Lewis R.
Lemann, Mrs. Benjamin
Lobb, Mr. and Mrs. John
Loeb, Dr. and Mrs. Robert F.
Lovell, Mr. and Mrs. Hollis R.
Makrauer, Mr. S. Lang
Marsland, Dr. and Mrs. Douglas A.
Marvin, Mrs. Walter Taylor
Mast, Mrs. S. O.
Mather, Mr. Frank J., III
McCusker, Mrs. Paul T.
McElroy, Dr. and Mrs. W. D.
McGillicuddy, Dr. and Mrs. John J.
McKelvy, Mr. John E.
McLane, Mrs. Huntington
McVitty, Mrs. A. E.
Meigs, Mr. and Mrs. Arthur
Meigs, Dr. and Mrs. J. Wister
Mellon, Mr. and Mrs. Richard P.

Minis, Mr. and Mrs. Abram J., Jr.
Mitchell, Mrs. James McC.
Mitchell, Mrs. Philip
Mixter, Mrs. William Jason
Mosser, Mrs. Benjamin D.
Motley, Mrs. Thomas
Newton, Miss Helen K.
Nichols, Mrs. George
Nims, Mrs. E. D.
The Aaron E. Norman Fund, Inc.
Packard, Mrs. Charles
Park, Mr. and Mrs. Malcolm S.
Parpart, Dr. and Mrs. Arthur K.
Pennington, Miss Anne H.
Philippe, Mr. Pierre
Putnam, Mr. William A., III
Redfield, Dr. and Mrs. Alfred C.
Reznikoff, Dr. and Mrs. Paul
Rivinus, Mrs. F. M.
Rudd, Mr. and Mrs. H. W. Dwight
Rugh, Mrs. Roberts
Sands, Miss Adelaide G.
Saunders, Mr. and Mrs. Lawrence
Shiverick, Mrs. Arthur
Sinclair, Mr. and Mrs. W. Richardson
Smith, Mrs. Homer P.
Speidel, Mrs. Carl C.
Stone, Mr. and Mrs. Leo
Stone, Dr. William, Jr.
Stone, Mrs. Samuel M.
Strauss, Mr. and Mrs. Donald B.
Stunkard, Mrs. Horace
Swope, Mr. David
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Szent-Györgyi, Dr. Albert
Tompkins, Mr. and Mrs. B. A.
Webster, Mrs. Edwin S.
Whiteley, Miss Mabel W.
Wickersham, Mrs. James H.
Wilhelm, Dr. and Mrs. Hilmer J.
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. W.
Yntema, Mrs. Chester L.

V. REPORT OF THE LIBRARIAN

The 1963 acquisitions added 68 new titles to our current journal collection, bringing the total number to 1826. Of this total, the Laboratory subscribed to 568, sent *The Biological Bulletin* in exchange for 644 and gratefully received 187 as gifts. The Woods Hole Oceanographic Institution subscribed to 144, sent its *Collected Reprints* in exchange for 215 and accepted 68 as gifts.

To our book shelves we added a total of 353 titles, the Laboratory having purchased 108 and the Institution 67. Generous authors contributed 11 books and 122 were presented by the publishers who held summer exhibits in the main lobby. From various other sources, 45 books were received.

Through purchase, exchange and gift the Laboratory completed 17 journal sets and partially completed 26 sets, whereas the Institution completed two sets and partially completed 9 sets. We added 4266 reprints to our collection, 2862 of which were of current issue.

There were 1089 volumes sent to the bindery. Outgoing inter-library loans totalled 306 and 76 titles were borrowed for our investigators. The Xerox machine installed by the Laboratory enabled us to give more efficient photoprint service to other libraries and to our own readers.

The Library now contains 82,436 bound volumes and 228,000 reprints.

Several gifts of books and reprints were received, for which the Librarian wishes to extend acknowledgment to Drs. Douglas A. Marsland, Roberts Rugh and Albert Szent-Györgyi. From the estate of Dr. Herbert W. Rand we acquired 60 books. We were extremely happy to get these, as many of them have been used to replace worn copies, many of which have been long out-of-print.

In November, work was started on Library alterations. These renovations cover extensive improvements in the stacks, new offices for the Staff, a rare-books room, a catalogue room and a photoprint room. It is anticipated that these changes will be advantageous to our readers and will increase the efficiency of the services rendered by the Staff.

Respectfully submitted,
DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1963, amounted to $2,155,489 as against book value of $1,242,896. This compares with values of $1,994,709 and $1,242,771, respectively, at the end of the preceding year. The average yield on the securities was 3.64% of the market value and 6.31% of book value. The total uninvested principal cash in the above accounts as of December 31, 1963, was $431.33. Classification of the Securities held in the Endowment Funds appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1963, was $390,026 with uninvested principal cash of $168.26, the market value at December 31, 1962, being $353,243. The book value of the securities in this account was $315,196 on December 31, 1963, compared with $302,453 a year earlier. The average yield on market value was 3.36% and 4.16% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1963, is as follows:

Pension Funds	31.117%
General Laboratory Investment	48.642
Other:	
Bio Club Scholarship Fund	1.393
Rev. Arsenius Boyer Scholarship Fund	1.705
Gary N. Calkins Fund	1.596
Allen R. Memhard Fund	.310
F. R. Lillie Memorial Fund	5.376
Lucretia Crocker Fund	5.819
E. G. Conklin Fund	.983
Jewett Memorial Fund	.517
M. H. Jacobs Scholarship Fund	.702
Anonymous Gift	1.840

Donations from the MBL Associates for 1963 were $4,295.00, as compared with $5,305 for 1962. Unrestricted gifts from foundations, societies and companies amounted to $24,990.

During the year we administered the following grants:

Investigators	*Training*	*MBL, Institutional*
11 NIH	5 NIH	4 NIH
4 NSF	2 NSF	3 NSF
1 Ford		3 ONR
1 Commonwealth		1 AEC
17	7	11

The rate of overhead on grants to investigators has been increased to 20%, based on the amount expended. The overhead on these grants for this year amounted to $56,494 as compared with $43,405 for the preceding year.

The Lillie Fellowship Fund, with a market value of $104,171 and a book value of $93,018, as well as the investment in the General Biological Supply House, with a book value of $12,700, is carried in the Balance Sheet item "Other Investments."

The General Biological Supply House fiscal year ended June 30, 1963, and had a profit after taxes of $241,616 as compared to $302,657 in 1962 and $302,851 in 1961 and $314,034 in 1960 and $303,300 in 1959. In the fiscal year 1963, the Marine Biological Laboratory received dividends from the General Biological Supply House of $42,164 as against $38,100 in 1962 and $33,020 in 1961 and $30,480 in 1960.

Following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as at December 31, 1963, the related statement of operating expenditures, income and current fund and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory for the year ended December 31, 1962.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1963 and 1962, and the results of its operations for the years then ended on a consistent basis.

Boston, Massachusetts
March 30, 1964

Lybrand, Ross Bros. & Montgomery
James H. Wickersham,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1963 and 1962

Investments	*1963*	*1962*
Investments held by Trustee:		
Securities, at cost (approximate market quotation, 1963—$2,155,000)	$1,242,896	$1,242,771
Cash	431	641
	1,243,327	1,243,412
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1963 $390,000) less $5,728 temporary investment of current fund cash	309,468	296,725
Other investments	120,424	139,677
Cash	14,083	15,135
Accounts receivable	21,131	31
	$1,708,433	$1,694,980
Plant Assets		
Land, buildings, Library and equipment (note)	4,931,472	4,900,749
Less allowance for depreciation (note)	1,313,162	1,247,149
	$3,618,310	$3,653,600
Current Assets		
Cash	100,991	118,732
Temporary investment in pooled securities	5,728	5,728
U. S. Treasury bills, due prior to 2/13/64 at cost	74,324	248,253
Accounts receivable (U. S. Government, 1963—$95,142; 1962—$41,579)	140,825	78,099
Inventories of specimens and Bulletins	45,288	41,903
Prepaid insurance and other	7,062	19,006
	$374,218	$511,721

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1963 and 1962

Endowment Funds	*1963*	*1962*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,243,327	$1,243,412
Endowment funds for awards and scholarships:		
Principal	126,980	126,302
Unexpended income	12,077	11,353
	139,057	137,655
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	108,481	94,048
Pooled investments—accumulated gain	11,190	13,487
	$1,708,433	$1,694,980

Plant Funds		
Funds expended for plant, less retirements	4,931,472	4,900,749
Less allowance for depreciation charged thereto	1,313,162	1,247,149
	$3,618,310	$3,653,600

Current Liabilities and Funds		
Accounts payable and accrued expenses	62,763	35,729
Advance subscriptions	10,362	7,524
Unexpended grants—research	110,433	344,437
Unexpended balances of gifts for designated purposes	13,531	9,461
Current fund	177,129	114,570
	$ 374,218	$ 511,721

Note.—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES, INCOME AND CURRENT FUND

Years Ended December 31, 1963 and 1962

Operating Expenditures

	1963	*1962*
Research and accessory services	$ 273,333	$ 237,580
Instruction	147,163	142,394
Library and publications (including book purchases—1963, $25,628; 1962, $24,287)	77,922	72,514
Direct costs on research grants	484,640	378,163
Direct costs on institution support grants	129,642	98,043
	1,112,700	928,694
Administration and general	97,339	92,124
Plant operation and maintenance	111,441	102,632
Dormitories and dining	174,030	155,234
Additions to plant from current income	7,793	22,088
	1,503,303	1,300,772
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	67,472	65,061
	1,435,831	1,235,711

Income

	1963	*1962*
Research fees	113,024	99,314
Accessory services (including sales of biological specimens—1963, $38,019; 1962, $29,898)	103,398	90,247
Instruction fees	28,461	27,439
Library fees, Bulletins, subscriptions and other	43,952	41,314
Dormitories and dining income	129,486	125,176
Grants for support of institutional activities:		
Instruction and training	131,564	127,988
Support services	129,642	98,043
General	113,853	79,500
Reimbursements and allowances for direct and indirect costs on specific research grants	541,134	421,568
Gifts used for current expenses	29,285	39,824
Investment income used for current expenses	134,591	120,697
	1,498,390	1,271,110
Excess current income	62,559	35,399
Current fund balance January 1	114,570	79,171
Current fund balance December 31	$ 177,129	$ 114,570

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1963

	Balance January 1, 1963	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1963*
Invested funds	$1,694,980	$ 19,897	$136,547	$ 127,580	$15,411	$1,708,433
Unexpended research grants	$ 344,437	682,189		916,193		$ 110,433
Unexpended gifts for designated purposes	$ 9,461	37,265		29,285	3,910	$ 13,531
Current fund	$ 114,570	62,559(1)				$ 177,129
Total gifts and other receipts		$801,910	$136,547	$1,073,058	$19,321	
Gifts		37,265				
Grants for research, training and support		682,189				
Net loss on sales of securities		(1,704)				
Appropriated from current income and other		21,601				
(1) Excess current income over expenditures		62,559				
Total		$801,910				
Scholarship awards					4,534	
Payments to pensioners					10,877	
Other					3,910	
					$19,321	

BIOCHEMICAL STUDIES ON THE COELOMIC FLUID OF NEPHTYS HOMBERGI (POLYCHAETA: NEPHTYIDAE), WITH OBSERVATIONS ON CHANGES DURING DIFFERENT PHYSIOLOGICAL STATES[1]

MARY E. CLARK

Department of Zoology, University of Bristol, Bristol, England

During investigations of regeneration in the polychaete, *Nephtys hombergi*, attempts have been made to culture tissues of this animal *in vitro*, using a modification of the medium used for insect tissues by Jones and Cunningham (1961), in order to study more closely the factors influencing regeneration. Although it was possible to obtain sterile cultures, and the survival, with occasional mitosis, of gut cells for more than three weeks, other tissues became degenerate and no true growth occurred. It was felt that lack of knowledge of the chemistry of the coelomic fluid of *Nephtys*, which bathes all the internal tissues, would hinder any attempts at further modifications of the culture medium.

Information on the biochemistry of polychaetes is still very patchy and incomplete. Studies on *Nephtys* include those of [illegible] and Labes (1951) on the gross chemical composition of whole animals, of Jones (1955) on its haemoglobin, of Hobson and Rees (1955) on its phosphagens and phosphokinases, and of Jollès *et al.* (1958) and Jollès and Zuili (1960) on a lysozyme occurring in *N. hombergi*. In no case has a general study of the biochemistry been undertaken.

This report deals with an exploratory investigation of the coelomic fluid, the major purpose being to obtain information useful to tissue culture and regeneration studies, and observations were made not only on normal worms, but also on actively regenerating animals and on those which are known to be incapable of regeneration, namely "brooding" animals (Clark and Clark, 1959; Clark *et al.*, 1962).

MATERIALS AND EXPERIMENTAL METHODS

Animals

Four batches of *Nephtys hombergi* were collected at fortnightly intervals from one area on the Severn Channel between late September and mid-November, 1962. The animals were placed in clean petri dishes containing autoclaved sea water with penicillin at 1000 units per ml., which was renewed daily. Such precautions are necessary as *Nephtys* easily succumbs to bacterial infections. Under these conditions animals have been kept healthy for more than a month at room temperature. No food was given during the experiment, because this hastens growth of contaminants, and in any case "brooding" animals are unable to feed.

Treatments

Each batch contained worms in a range of sizes, although animals too small to bleed were not collected. Each batch was divided into 3 groups having equal proportions of large to small animals, and the groups were treated as follows: (1) intact controls, starved one day (*i.e.,* bled the day following collection) = *S*-1 (2) animals with the caudal third of the body ("tail") amputated on the day after collection, as described by Clark and Clark (1962), and bled three days later = *T*-3; (3) animals with "tails" amputated as above but allowed to regenerate for ten days = *T*-10; (4) animals with the supraoesophageal ganglion (= "brain") removed on the day following collection and bled 10 days later = $\bar{B}$-10; (5) animals starved 11 days but otherwise intact = *S*-11. The three-day regenerating group was included because this is the day on which large amounts of brain hormone are released in the closely related genus, *Nereis diversicolor* (Clark and Ruston, 1963) and thus is the moment when specific effects due to the hormone might be expected.

---

[1] This work was done during tenure of a U.S. Public Health Service postdoctoral research fellowship (GF-11,847).

Brain removal was carried out aseptically after anesthesia in 0.1% MS 222 in sea water. Between 80% and 90% of such operated animals survived the experimental period.

Removal of coelomic fluid

For the sake of brevity, the term "bleeding" has been used throughout this paper instead of the cumbersome "removal of coelomic fluid," but in fact, the amounts of blood contaminating the samples were extremely small and are unlikely to have had an effect on the results.

The worms were killed by brief immersion in 95% ethanol, rinsed in sea water, blotted on filter paper to remove external moisture and placed on a clean glass surface. Coelomic fluid was removed by successive punctures along the length of the body with a capillary pipette. It sometimes happened that blood or gut fluid was also drawn up, and these were both discarded. *Nephtys* blood is readily detected by its brilliant red color, while gut fluid is brown or black and contains a surface-active agent that causes excessive frothing.

Coelomic fluid from all worms in each group was pooled. The pinkish-red liquid, colored by its own hemoglobin (Jones, 1955), was transferred immediately after bleeding into chilled 15-ml. centrifuge tubes, and stored at −17° C.

Chemical methods employed are described with the results.

Chemical Methods and Results

In Table I is given a list of the samples of coelomic fluid used in this study. Each sample was centrifuged 10 minutes at 3000 RPM before use to remove the formed elements, such as gametes, sloughed muscle fibers and coelomocytes.

Precipitation of high-molecular-weight constituents was usually accomplished by addition of 5 volumes of 96% ethanol to aliquots of coelomic fluid. After standing for 15 minutes in the cold to complete flocculation, these were centrifuged as above.

In all colorimetric studies, duplicate determinations on each coelomic fluid sample were made where sufficient material was available.

pH and buffering capacity

It was not possible to measure the pH of *Nephtys* coelomic fluid immediately after removal from the animals as it took too long to collect sufficient material

TABLE I

Coelomic fluid samples

Batch and date collected	Sample treatment	No. of worms	Ml. of pooled sample	pH*
I	S–1 Starved 1 day	57	0.7	6.40
28.ix.62	$\bar{T}$–3 Tailless 3 days	60	1.0	6.70
	S–11 Starved 11 days	61	1.0	6.50
	$\bar{T}$–10 Tailless 10 days	77	0.9	6.52
	$\bar{B}$–10 Brainless 10 days	60	0.7	6.40
II	S–1 Starved 1 day	40	1.0	6.60
12.x.62	$\bar{T}$–3 Tailless 3 days	63	0.9	6.78
	S–11 Starved 11 days	41	1.0	6.60
	$\bar{T}$-10 Tailless 10 days	61	0.9	6.60
	$\bar{B}$–10 Brainless 10 days	51	0.5	6.40
III	S–1 Starved 1 day	45	1.0	6.50
25.x.62	$\bar{T}$–3 Tailless 3 days	56	0.9	6.88
	S–11 Starved 11 days	33	0.7	6.58
	$\bar{T}$–10 Tailless 10 days	52	0.9	6.60
	$\bar{B}$–10 Brainless 10 days	41	1.1	6.62
IV	S–1 Starved 1 day	36	0.9	6.42
9.xi.62	$\bar{T}$–3 Tailless 3 days	38	1.0	6.70
	S–11 Starved 11 days	31	1.0	6.40
	$\bar{T}$–10 Tailless 10 days	38	0.9	6.40
	$\bar{B}$–10 Brainless 10 days	47	1.2	6.40
	S–3 Starved 3 days	24	0.7	6.72
2.ii.64	$\bar{T}$–3 Tailless 3 days	27	0.3	6.94

* *In vivo* pH values probably slightly lower; see text.

to cover the relatively large glass electrode available, so measurements were made on pooled samples equilibrated with air. The pH values, measured with an E.I.L. direct-reading pH meter, are also given in Table I. The pH range of normal animals is 6.4–6.6. This may be slightly higher than the *in vivo* values, due to CO$_2$ loss from the samples before measurement. It will be seen that the pH of the coelomic fluid of three-day regenerating animals ($\bar{T}$-3) rises by 0.2–0.3 unit, but that it returns to the normal value later ($\bar{T}$-10). The other samples did not show significant or consistent variation from intact control animals. To determine whether the pH rise seen in three-day regenerating worms was in fact due to regeneration or was only the result of a short period of starvation, a further group of regenerating animals was compared with a three-day starved group (Table I), and again a pH rise of about 0.2 unit was found in regenerating animals.

The buffering capacity of a sample of coelomic fluid collected in August (not listed in Table I) was determined by electrometric titration. Each 100-μl. aliquot was diluted with 10 ml. 0.5 *M* NaCl and titrated with standardized 10^{-3} N HCl and

10^{-3} N NaOH, using a Doran pH meter. Dilution in 0.5 *M* NaCl was used to maintain approximately physiological ionic strength, shown to be important in determining the buffering capacity of proteins by Redfield and Ingalls (1932). Subtraction of "blank" titration values on aliquots of 0.5 *M* NaCl alone gave the results shown in Figure 1. The buffering capacity between pH 4.5 and 7.5 is about 8 meq./1./pH unit.

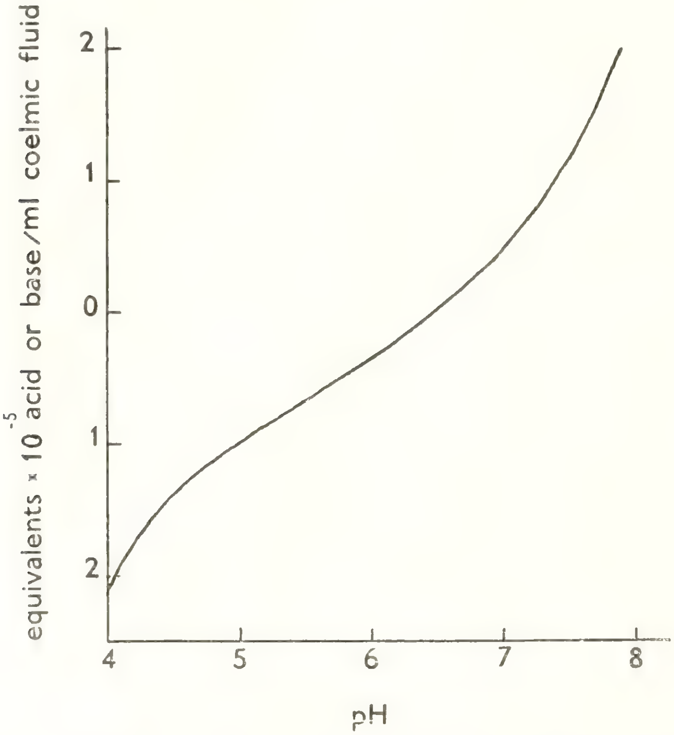


FIGURE 1. Curve of buffering capacity of *Nephtys* coelomic fluid. One hundred μl. diluted in 10 ml. 0.5 *M* NaCl and titrated with 10^{-3} N HCl and NaOH.

Carbohydrate distribution

Total carbohydrate was determined colorimetrically in whole coelomic fluid and in protein-free filtrates by reaction with anthrone according to the method of Fong *et al.* (1953), but using 2 ml. of anthrone reagent and a final volume of 5 ml. When either tungstic acid or trichloracetic acid were used as protein precipitants they were found to interfere with the reaction (see Seifter *et al.*, 1950), whereas precipitation in 80% ethanol did not, and this was the method employed. Glucose standards of 12.5, 25, 50 and 100 μg. were run with every set of unknowns, thus taking into account the slight and fairly reproducible deviation from Beer's Law at higher concentrations. As will be seen, the range of values among the coelomic fluid samples was very great.

Individual sugars were estimated by paper chromatography. Aliquots of coelomic fluid (usually 0.15 to 0.20 ml.) were deproteinized with $ZnSO_4$ and $Ba(OH)_2$ (Somogyi, 1945). There was only sufficient material for one chromatogram of each sample. Salts were removed using the method of Britton (1962). Two grams of Bio-Deminrolit were used for each sample, the resin first being saturated with CO_2 to prevent adsorption of reducing sugars (Woolf, 1953). After two 2-ml. rinses of the column with water the total effluent was concentrated *in vacuo* and spotted onto a strip of Whatman No. 1 filter paper. On each paper, standards ranging from 5 to 100 μg. of raffinose, maltose, sucrose, glucose, ribose and fructose were also spotted. Good separation was obtained by running descending "Durchlauf" chromatograms in the upper phase of n-butanol:acetic acid:water (40:10:50) for 24 hours at 25° C., and the spots were located with the p-anisidine

TABLE II

Carbohydrate distribution in coelomic fluid samples. Anthrone estimations expressed as mg. glucose equivalents per 100 ml., other sugars as mg. per 100 ml. Alcohol-precipitable carbohydrate was obtained by difference. O = no visible spot. + = spot present but not estimated

		Anthrone estimations			Chromatography of free sugars					
Batch and date collected	Treatment	Total CH_2O	Alc-sol. CH_2O	Alc-ppt. CH_2O	Glucose	Maltose	"Triose"	Ribose	Sucrose	Total
I 28.ix.62	S–1	174	99	75	70	50	2	0	0	122
	$\bar{T}$–3	120*	103*	17	64	60	2	0	0	126
	S–11	107	80	27	81	9	1	+?	0	91
	$\bar{T}$–10	133	82	51	85	10	+?	0	0	95
	$\bar{B}$–10	196	18	178	98	61	2	0	0	161(?)
II 12.x.62	S–1	264	268	"0"	—	—	—	—	—	—
	$\bar{T}$–3	152	112	40	26	44	1	0	0	71
	S–11	108	48	60	—	—	—	—	—	—
	$\bar{T}$–10	72	60	12	22	4	0	0	0	26
	$\bar{B}$–10	—	180†	—	54	102	2	0	0	158
III 25.x.62	S–1	196	192	4	51	53	2	0	0	106
	$\bar{T}$–3	76	44†	32	30	13	1	+	0	44
	S–11	128	28†	100	47	93	+?	0	0	140(?)
	$\bar{T}$–10	92	84	8	39	23	1	0	0	63
	$\bar{B}$–10	168	122	46	23	77	2	0	0	102
IV 9.xi.62	S–1	165	141†	24	75	70	2	0	0	147
	$\bar{T}$–3	81	57†	24	25	4	0	0	3	32
	S–11	154	69	85	63	30	2	0	0	95
	$\bar{T}$–10	130	79	51	74	53	2	0	0	129(?)
	$\bar{B}$–10	250	149†	101	86	78	5	0	0	169

† Only one determination possible.

* Poor agreement of replicate determinations.

(?) Unexplainable discrepancies from anthrone measurements.

phosphate reagent of Mukherjee and Srivastava (1952). The minimum sensitivity for the sugars present was between 1 and 5 μg. Sugars in the coelomic fluid samples were semi-quantitatively estimated independently by five persons, by visual comparison with the standard spots, and the results averaged.

The results of carbohydrate determinations are given in Table II, and those of anthrone determinations are also presented graphically in Figure 2. The total sugars estimated on the semi-quantitative chromatograms are mostly in the same range as those found in the alcohol-soluble fraction of the corresponding sample with the anthrone reagent. Only in one case, Batch III, *S*-1, is it likely that a large quantity of sugar may have been undetected. Incomplete recovery from desalting columns may account for those discrepancies where chromatogram totals are considerably lower than anthrone estimates, but the three cases where the chromatographic estimate is very much higher (indicated by "?" in Table II) are harder to explain. The anthrone estimates, except as noted, were run in duplicate and agreement was as follows: 18 better than 10%; 9 worse than 10% but better than 20%; 3 worse than 20% but better than 30%; and 4 worse than 30%. Values for alcohol-precipitable carbohydrate were obtained by difference and bear the same uncertainty as the parent figures from which they were derived.

An attempt to identify the alcohol-precipitable carhohydrate was made by treating an ethanol-washed and -dried preparation with β-amylase (*ex* barley: L. Light), and then chromatogramming the deproteinized, desalted sample by the method described above. No neutral sugars whatever were found, and the alcohol-precipitable carbohydrate is thus not glycogen.

The chromatograms revealed three spots on nearly all samples of coelomic fluid. One of these was clearly glucose, and the other major component had the same R_F value and color reaction as maltose. This identification was supported by finding that the spot disappeared and the glucose spot increased after treatment of an alcohol-soluble preparation with yeast maltase (L. Light). The third spot, always very faint and yellow-brown in color, was very slow-moving. It behaved similarly to raffinose in these respects, but no further identification has been attempted. In addition, very small amounts of ribose occurred in two samples and sucrose in another (as identified by their R_F values and color reactions, only).

It will be seen in Table II that in fed animals glucose and maltose occur in about equal amounts, but during starvation maltose levels often fall more than those of glucose. A consistent exception is the brainless condition, in which maltose is maintained at or above normal levels.

Nitrogen distribution

The total nitrogen content of both whole coelomic fluid and 80% ethanol-soluble filtrates was determined colorimetrically by direct Nesslerization using the micromethod of Weed and Courtenay (1954). Estimates were made on 2.5- or 5.0-μl. aliquots of coelomic fluid samples. (It was found that better agreement with Beer's Law could be obtained when the H_2SO_4 used for digestion was neutralized by replacing the 4 ml. of 0.01% chilled gum ghatti solution of the original method with a mixture of 2 ml. 0.9 N NaOH and 2 ml. 0.02% gum ghatti. This modification was used with Batches I and IV.) Alcohol-precipitable nitrogen was ob-

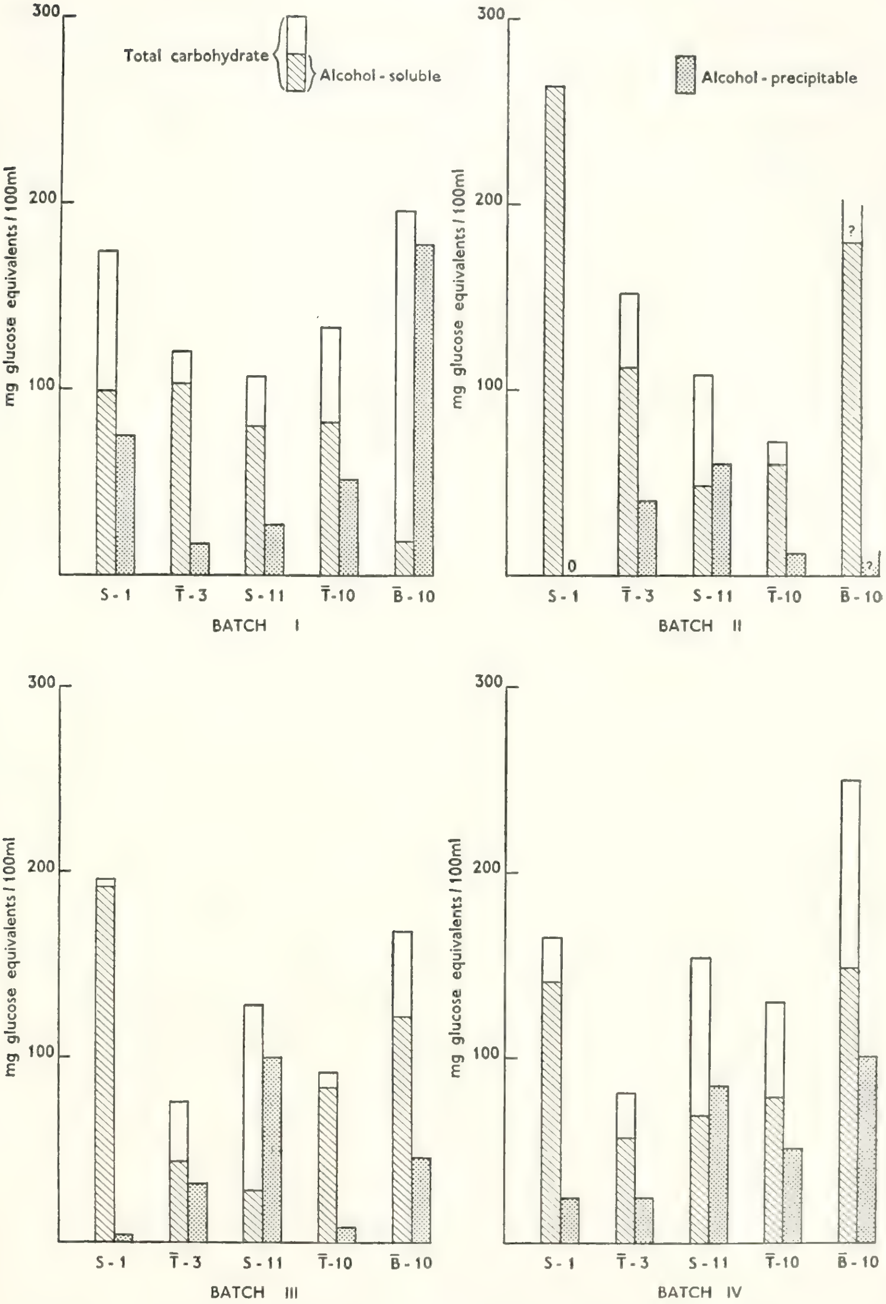


FIGURE 2. Carbohydrate distribution in *Nephtys* coelomic fluid, estimated by anthrone determinations on whole coelomic fluid and the alcohol fraction. See Table I for description of batches and treatments.

tained by difference. In addition, direct estimates on twice-washed aliquots of the alcohol-precipitable fraction of Batch IV samples were carried out as a check.

Estimates of total amino acid nitrogen were made on aliquots of alcoholic filtrates equivalent to 2.5 or 5.0 μl. whole coelomic fluid of Batches I and IV. A very reproducible ninhydrin micro-method developed by A. P. Sims and D. K. Lewis (personal communication) was used. Briefly, it is as follows: stock solutions are (1) citrate buffer, pH 4.8 (42 g./l. citric acid + 16 g./l. NaOH for single strength) and (2) 11.5 g. ninhydrin dissolved in redistilled methyl cellosolve. Immediately before use, 10 ml. of the ninhydrin stock are added to 4 ml. of a 1% w/v aqueous ascorbic acid solution, and the mixture is made to 120 ml. with methyl cellosolve. To 1 ml. of sample containing amino acids are added 0.5 ml. adjusting buffer and 1.2 ml. final ninhydrin solution. The covered tubes are heated at 100° C. for 20 minutes, cooled 5 minutes in tap water and diluted with 3 ml. 60% ethanol. Optical density is read at 570 mμ, 0.27 O.D. units being equivalent to 1 μg. amino nitrogen. The reaction is specific for α-amino nitrogen and ammonia, other nitrogenous compounds interfering very little.

Measurements were also made of the amount of α-amino nitrogen released on hydrolysis of 2.5-μl. aliquots of whole coelomic fluid samples. To see whether some of this might be due to alcohol-soluble peptides, aliquots of the alcohol-soluble filtrates of Batches I and IV were also hydrolyzed before being treated with ninhydrin, and afterwards compared with unhydrolyzed samples. As a check on accuracy, protein nitrogen was directly analyzed with ninhydrin after hydrolysis of the alcohol-insoluble fraction of samples in Batch IV. Hydrolyses were carried out in sealed Pyrex tubes, similar to those used for Nessler digestions (Weed and Courtenay, 1954), containing usually 8 volumes of 1 N H_2SO_4. Hydrolysis was effected at 15 lb. pressure for 4 hours (further hydrolysis yielded no increase in ninhydrin-positive reactants). The hydrolysis tubes were broken into large test tubes into which the other reagents were pipetted. Following prolonged mixing, the reaction was carried out as above. For the direct protein nitrogen estimates on Batch IV, alcoholic precipitation was carried out in small Pyrex tubes, followed by centrifugation. The residue, washed twice with 80% ethanol, was taken to dryness. After hydrolysis, aliquots equivalent to 5 μl. coelomic fluid were transferred to the final reaction tubes. When the ninhydrin reaction was employed on these hydrolyzed samples, double-strength citrate buffer was used, since it is essential to reproducibility that the pH of the reaction be kept within narrow limits.

Amino acid chromatograms were carried out on 50–200-μl. samples of whole coelomic fluid deproteinized and desalted by the one-step method of Harris, Tigane and Hanes (1961), with slight modifications. Resin desalting, rather than alcohol precipitation, was found essential for obtaining clear chromatograms. For complete retention of the acidic amino acids it was necessary to treat the resin with 3 N HCl before each use. Acidification of the sample prior to putting it on the column caused a precipitate to form which was removed by filtration. Two 1.0-ml. acetic acid rinses and one 1.5-ml. distilled water rinse were needed to remove all the inorganic anions from the resin. Elution was carried out with 3.0 ml. 2.5 N triethylamine in 20% acetone. Even with this higher concentration of base, only 50–70% of the arginine could be recovered from standard mixtures when the separation was similar to that in a 100-μl. sample (2–6 μmoles total amino acids from about 60

TABLE III

Nitrogen distribution in coelomic fluid samples, expressed as mg. nitrogen per 100 ml.

Nessler estimations						Ninhydrin estimations						Chromatography
1	2	3	4	5	6	7	8	9	10	11	12	13
				Alc. ppt. N			Alc. sol. N			Alc. ppt. N		
Batch and date collected	Treatment	Total N	Alc. sol. N	(by diff.) (3–4)	(direct)	Total N +HCl	−HCl (α amino +NH_3)	+HCl (α amino +NH_3 +peptide)	Peptide N (by diff.) (9–8)	(by diff.) (7–9)	+HCl (direct)	Amino N
										(protein)		
I	*S*-1	305	222	83	—	138	80	90	10	48	—	64
28.ix.62	$\bar{T}$-3	356	260	96	—	147	79	95	16	52	—	71
	S-11	372	274	98	—	143	96	104	8	39	—	62
	$\bar{T}$-10	388	262	126	—	—	85	97	12	—	—	62
	$\bar{B}$ 10	436	344	92	—	175	113	110	"0"	62	—	92
II	*S*-1	407	261	146	—	—	—	—	—	—	—	48
12.x.62	$\bar{T}$-3	345	223	122	—	—	—	—	—	—	—	51
	S-11	337	122	215	—	—	—	—	—	—	—	43
	$\bar{T}$-10	287	208	79	—	—	—	—	—	—	—	48
	$\bar{B}$-10	346†	259	87	—	—	—	—	—	—	—	54
III	*S*-1	259*	192	67	—	—	—	—	—	—	—	43
25.x.62	$\bar{T}$-3	250*	157	93	—	—	—	—	—	—	—	33
	S-11	381*	50	331	—	—	—	—	—	—	—	48
	$\bar{T}$-10	311*	201	110	—	—	—	—	—	—	—	48
	$\bar{B}$-10	203*	183	20	—	—	—	—	—	—	—	37
IV	*S*-1	279	178	101	82	128	79	92	13	36	34	53
9.xi.62	$\bar{T}$-3	192*	113	79	71	117	69	90	21	27	27	42
	S-11	299	192	107	63	145	82	110	28	35	30	59
	$\bar{T}$-10	319	246	73	68	148	89	117	28	31	28	66
	$\bar{B}$-10	351	275	76	71	168	101	132	31	36	29	70

† Only one determination possible.

* Poor agreement of replicate determinations.

μeq. cations). After evaporation the entire sample was dissolved in water and spotted onto 28 × 30 cm. sheets of Whatman No. 1 paper. Ascending development was used in the solvents of Redfield (1953). The amino acids were located and quantitatively estimated by the method of Wellington (1952, 1953). Blank spots were cut from various levels of the chromatogram to take into account variations in the intensity of background color in the direction of the second solvent. All spots at each level including the blank were cut to the same size as the largest spot to eliminate errors due to differences in area of the spots. After elution, the optical density of the spots was read at 570 mμ, with the spectrophotometer set at zero absorption for the appropriate blank. Standard curves were prepared from chromatograms spotted with 20 μg., 10 μg. or 5 μg. of each of the amino acids. However, it was found that when a known mixture of amino acids of the same proportions as found in *Nephtys* coelomic fluid was chromatogrammed, the values obtained using these standard curves deviated from the correct values in a reproducible manner, and correction factors were then applied to the results. The disproportionately large amounts of glycine may have been responsible for these deviations, which seemed to affect the background intensity in both directions.

In all but one case there was sufficient coelomic fluid to run two or more chromatograms. A second chromatogram was used for estimation of proline, which overlaps alanine and tyrosine in these solvents. The proline spot, together with overlapping spots, was cut from the chromatogram after color development in the usual way, and developed with acidified ninhydrin by the method of Chinard (1952) as modified by Wyatt *et al.* (1956). Neither oxidation of the spotted sample (Dent, 1948) in 10 instances, nor spraying with the platinic iodide reagent of Toennies and Kolb (1951) in 5 others, gave any indication of a cysteine spot, although methionine was detectable at 3.5 μg. with the latter. It is probable that if cysteine is present as a free amino acid, it was removed by the resin during desalting, since in desalted standards cysteine also was poorly recovered.

Estimation of glutamine and asparagine was carried out on eight samples where there was sufficient coelomic fluid. A desalted, evaporated aliquot was hydrolyzed with about 1 ml. 1 N HCl for two hours at 100° C. The HCl was removed *in vacuo* over saturated NaOH, and the sample spotted on as before. The increases in glutamic and aspartic acids gave estimates of these two amides. When glutamine was not estimated separately, it was included with the glycine spot with which it overlapped considerably. Histidine also was obscured by the very large glycine spot, and values reported are uncertain.

Two or more estimates of most amino acids from each sample were carried out. The average of duplicate runs (including desalting) of the artificial mixture made similar to *Nephtys* coelomic fluid gave readings within 20% of the expected value for most amino acids. Regular exceptions were arginine and lysine, due to variable and incomplete elution, threonine whose spot was often poorly delimited, and tryptophan, which was present only at low concentration near its limit of sensitivity. Total recovery of amino acid nitrogen was 93% or better.

The results of the nitrogen determinations are given in Table III and Figure 3. A typical chromatogram of *Nephtys* coelomic fluid is shown in Figure 4 b. Except for the spot at X-1, which appeared on most of the chromatograms, no unidentified spots occurred. X-1 did not disappear from samples after the mild hydrolysis used

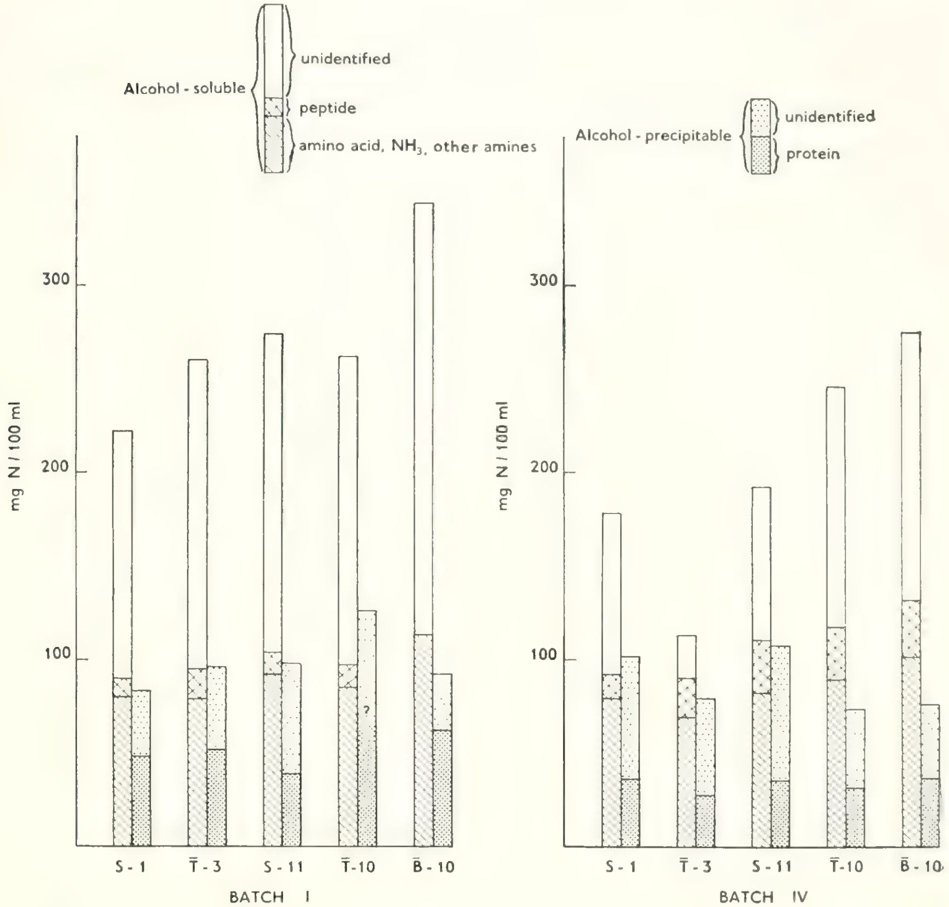


FIGURE 3. Nitrogen distribution in *Nephtys* coelomic fluid, estimated by direct Nesslerization and by ninhydrin determinations. See text for details, and Table I for description of batches and treatments.

for asparagine and glutamine, but no further attempts at identification were made. Quantitative results are given in Table IV. The values for X-1 were calculated from the standard curve for glycine and are thus an underestimate.

Where direct measurements of alcohol-precipitable nitrogen were carried out to confirm values estimated by difference, the agreement is reasonably good, especially with ninhydrin estimations. The difference between the amino nitrogen found by quantitative chromatography and the nitrogen estimated by direct ninhydrin reaction on the alcohol-soluble fraction may be due to several factors, which will be discussed later.

DISCUSSION

Before considering the results reported here, it is necessary to comment on the state of the animals used in these experiments. It was originally intended that the

TABLE IV

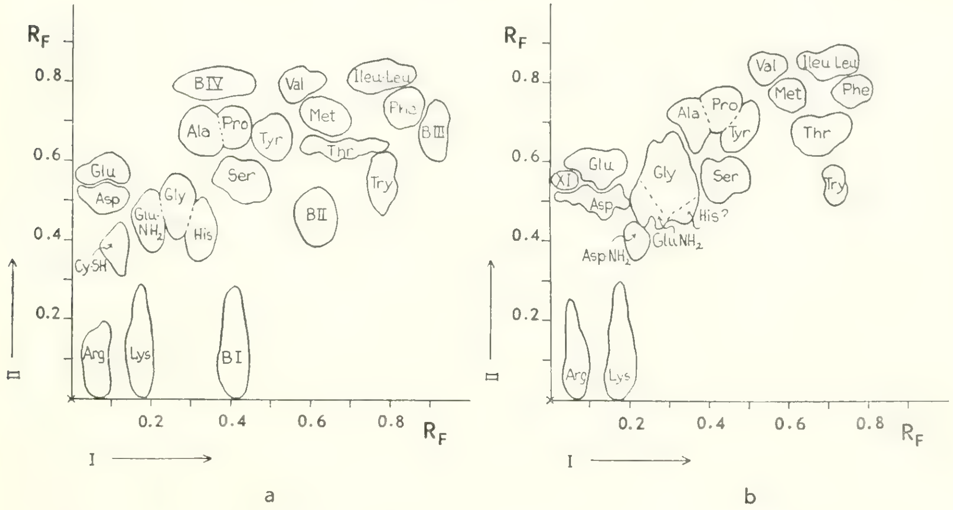
Free amino acids of coelomic fluid expressed as mg. per 100 ml. 0 = no visible spot. + = present but not estimated. ? = uncertainty due to overlap. — = no observation.

Treatment	Batch I					Batch II					Batch III					Batch IV				
	S-1	$\bar{T}$-3	S-11	$\bar{T}$-10	$\bar{B}$-10	S-1	$\bar{T}$-3	S-11	$\bar{T}$-10	$\bar{B}$-10†	S-1	$\bar{T}$-3	S-11	$\bar{T}$-10	$\bar{B}$-10	S-1	$\bar{T}$-3	S-11	$\bar{T}$-10	$\bar{B}$-10
Arginine	22	10	10	16	17	8	20	5	6	11	27	19	19	14	12	18	18	28	35	36
Lysine	20	12	10	14	18	15	25	7	10	18	27	19	28	17	12	23	18	25	29	31
Histidine	6	6	0?	0?	22	9	7	0?	0?	0?	0?	0?	+ ?	0?	0?	15	0?	17	11	19
Aspartic acid	26	29	33	28	35	22†	41	38	41	56	29	29	29	39	27	18	22	23	29	32
Asparagine	+	11†	0†	16†	6†	+	—	—	—	—	+	+	+ ?	+	+	22†	21†	21†	10†	7†
Glutamic acid	20	18	18	16	25	21	17	12	12	20	18	18	16	14	15	17	17	20	30	32
Glutamine		8†	4†	9†	8†		11†									15†	11†	20†	4†	7†
Glycine	185*	222	211	190	280	148*	132	147*	174*	167*	97*	91*	128*	141*	102*	135	90	122	161	164
Alanine	34	40	27†	27	40	23†	23	19†	19	27	26	3	19	20	21†	29	22	26	27	43
Valine	8	3	3	3	6	7	8	3	4	7	9	9	10	6	5	6	6	9	11	10
Isoleucine + leucine	22	12	11	13	24	16	26	7	11	22	30	25	31	18	11	26	24	35	38	38
Serine	17	10	8	11	15	9	9	6†	7	7	9	2	10	10	9	13	11	13	19	15
Threonine	21	17	15	21	24	8	11	5	8	9	7	3	11	11	6	12	14	18	28	17
Proline	42†	63†	38†	47†	78†	31†	21†	24†	11†	+	15†	18†	21†	19†	26†	16†	12†	41†	20†	39†
Phenylalanine	11	8	7	6	12	7	8	3	5	10	23	8	15	10	6	14	16	23	20	17
Tyrosine	13	9	10†	9	14	6†	9	4†	5	10	10	5	9	9	6†	13	13	13	19	17
Tryptophan	3	2	1	2	3	1	2	0	0	2	2	2	2	0	1†	2	2	3	2	3
Methionine	5	4	3	3	4	6	8	3	4	9	8	2	7	4	4†	5	7	7	8	7
X-1	5	5	4	4	7	3	8	2	2	3	7	5	11	6	7	6	4	12	12	9
Total	460	489	413	435	638	340	386	285	319	378	344	258	366	341	270	405	328	476	513	543
Mg. amino acid N/100 ml.	64	71	62	62	92	48	51	43	48	54	43	33	48	48	37	53	42	59	66	70
Millimoles/liter	46	51	44	44	66	34	36	31	35	38	31	24	34	34	26	38	30	42	47	50
Mole fraction glycine	0.53	0.58	0.63	0.57	0.56	0.57	0.48	0.64	0.67	0.58	0.41	0.50	0.48	0.56	0.51	0.47	0.40	0.38	0.45	0.43

† Based on one estimate only.

* Includes glutamine spot.

four batches of animals should serve as replicates, but subsequent histological examination (unpublished) of *Nephtys* collected at different times during the year revealed that most animals in September still retain mature gametes. During October and November these residual gametes are resorbed and by December this process is usually completed. Thus, Batch I was collected near the end of the breeding season, and Batches II, III and IV at successive stages during gamete resorption. Since by mid-winter the gonads show clear signs of preparation for the next breeding season there is no period when the animals are not in a state of flux with regard to their breeding biology. The ability of *Nephtys* to regenerate posterior segments at different times of the year has not yet been worked out precisely as it has for the closely related genus, *Nereis,* where it is clear that animals which have reached a certain degree of maturity can no longer regenerate (Clark and Ruston, 1963; Scully, 1964). It is likely that regenerative capacity in *Nephtys* is least during the breeding season, and it is known that brain removal, which induces precocious sexual maturity, prevents regeneration (Clark and Clark, 1959; Clark *et al.*, 1962). The added variable of seasonal differences makes it more difficult to interpret the experimental data, and in certain areas the discussion will necessarily be of a speculative nature.



a

b

FIGURE 4. Tracings of typical amino acid chromatograms, reduced. a, Standard mixture. B. I–IV are blank spots used for quantitative estimation. b, Desalted coelomic fluid of *Nephtys.* Solvent I: *tert*-butanol, methyl-ethyl ketone, diethylamine, water (40:40:4:20). Solvent II: methanol, pyridine, water (80:4:20).

Two sources of error which remained unassessed were variations in the nutritional state of the total population from one collecting period to the next, and large variations between individual animals within each batch. The first would be difficult to control except by artificially feeding the worms after collection, but its effects might be detected by studying variations between batches collected at shorter intervals. The fortnightly collecting times, however, fell at equivalent times in the tidal cycles. In the second case, although differences due to extreme variations between individual animals could only be detected by replicating experimental groups of worms, at least 30 animals contributed to each sample of coelomic fluid and no individual contributed more than 15% of the total, and hence a significant error from this cause is unlikely. The limited number of animals available was therefore spread over several experimental groups rather than used for replication.

One other possible source of error requiring comment is the presence in the coelomic fluid of enzymes which might break down some of the polymeric compounds. Material was kept frozen until determinations were made, although centrifugations and measurements of aliquots were done at room temperature. Evidence from homogenates of whole *Drosophila* (Mitchell and Simmons, 1962) shows that large changes may take place in a very few minutes in that species, and at present some enzymatic activity cannot be ruled out here (*e.g.,* Jollès *et al.,* 1958; Jollès and Zuili, 1960). However, as all samples within a given batch were handled together, differences between them must either indicate differences in the concentrations of compounds or in enzymatic activity.

Biochemical Aspects

Hydrogen ion concentration and buffering capacity

The pH of the body fluids of marine invertebrates is usually more acid than sea water, generally ranging between 7.2 and 7.8 (Prosser and Brown, 1961, p. 62). Smith (in Cole, 1940) found the pH of the coelomic fluid of the echiuroid, *Echiurus pallasii,* to be 7.6 and of the blood of the polychaetes, *Amphitrite brunnea* and *Glycera dibranchiata,* to be 6.8 and 7.4, respectively. (In the latter two cases, it is probable the measurements were actually of coelomic fluid, which is pigmented in both species.) The pH of *Nephtys* coelomic fluid is well on the acid side of neutrality despite the fact that samples were exposed to air. In this it resembles the hemolymph of insects, where, in most species, the pH lies between 6.4 and 6.8 (Heimpel, 1955, 1956; Wyatt *et al.,* 1956).

The buffering capacity of *Nephtys* coelomic fluid of about 8 meq./l./pH unit is in the upper part of the range for invertebrates. That of *Limulus* blood is 10 meq./l./pH unit, due primarily to its hemocyanin content (Redfield *et al.,* 1929). That of *Urechis caupo* coelomic fluid is 4.9 meq./l./pH unit and is accounted for entirely by the hemoglobin (1.8–4.0 g./100 ml.) (Redfield and Florkin, 1931). That of *Busycon canaliculatum* is 3.4 meq./l./pH unit (calculated from Florkin, 1934) and of *Echinus* (Pantin, in Barcroft, 1934, and personal communication) is approximately 3 meq./l./pH unit. According to Prosser and Brown (1961, p. 231) most of the buffering power of invertebrate body fluids resides in the proteins, particularly the pigments. Although the blood of *Nephtys* contains a high concentration of hemoglobin which may, in fact, bind considerable CO_2, the buffering capacity of the coelomic fluid is unlikely to be due to the content of hemoglobin found there (see below). Even the total protein is only 0.2 to 0.4 g./100 ml. and it is likely that the buffering is mainly due to the free amino acid pool of 30–45 mmoles/l.

Carbohydrates

The total carbohydrate of *Nephtys* coelomic fluid is composed of two fractions, one the alcohol-soluble sugars, the other an alcohol-insoluble moiety. The latter is present in concentrations too high merely to be an artifact due to interference of the anthrone reaction by proteins or amino acids (Seifter *et al.,* 1950). Whether or not the alcohol-precipitable fraction is a single substance remains to be seen. There does not appear to be any previous report of a high-molecular-weight carbohydrate in the coelomic fluid of annelids, although glycoproteins probably occur in the hemolymph of some insects (see Wyatt, 1961), and Meenakshi and Scheer (1961) report a large proportion of acid-soluble glycoprotein in crab blood.

Information on sugars in marine annelids is also scanty. Seton and Wilber (1949) report normal coelomic glucose levels in *Amphitrite ornata* at around 32 mg./100 ml. and in the sipunculid, *Phascolosoma* (= *Golfingia*) *gouldii,* at 17 mg./100 ml. The method of estimation is not given and is presumably based on reducing activity, in which case other sugars may be included. The level of alcohol-soluble carbohydrate found in *Nephtys* is considerably higher, and shows a wide range, from 100 to 260 mg./100 ml. This variation may be partly seasonal, with a marked increase just after breeding, and lower levels later in the year. Glucose values alone lie between 50 and 75 mg./100 ml., higher than in most other invertebrates investigated. Insects, except Hymenoptera, have very little glucose, the major blood sugar being trehalose (see discussion by Wyatt, 1961) and the amounts in crustaceans are also lower, being between 2 and 23 mg./100 ml. in various species (Kleinholz and Little, 1949; Scheer and Scheer, 1951; Meenakshi and Scheer, 1961).

The occurrence of maltose has not previously been reported in polychaetes, although in crabs maltose, as well as maltotriose and maltotetrose, has been reported in amounts similar to total glucose levels (Meenakshi and Scheer, 1961). A disaccharide, identified as trehalose, has been found in whole body extracts of several polychaetes (Fairbairn, 1958). The third, faint spot which appeared on most of the chromatograms has such a low mobility as to suggest that it is a higher order oligosaccharide. Amino-sugars, sugar acids and sugar phosphates, if present, would have escaped detection due to removal during the desalting process (Britton, 1962). The possibility, however, of undetected sugars regularly occurring in large amounts in *Nephtys* coelomic fluid appears unlikely in view of the correspondence between chromatogram estimates and anthrone measurements in most cases.

Nitrogen metabolism

The total free amino acids in *Nephtys* coelomic fluid lie between 350 and 450 mg./100 ml. There are no previous data on polychaete body fluids for comparison. Among marine arthropods, values from 5 mg./100 ml. to 64 mg./100 ml. have been reported (Stevens *et al.,* 1961; Camien *et al.,* 1951). The echiuroid, *Urechis caupo,* has about 80 mg./100 ml. (Giordano *et al.,* 1950). Amino acid levels in insect hemolymph are much higher, the hemimetabolous species ranging from 275 mg./100 ml. to 350 mg./100 ml. (Stevens, 1961; Duchâteau and Florkin, 1958), while the holometabolous forms have very high values, around 850–1500 mg./100 ml. (Wyatt *et al.,* 1956). *Nephtys* thus lies near the hemimetabolous insects in the amount of free amino acids in its body fluid, and is the only other invertebrate so far studied which approaches insects in this respect.

The individual amino acids often have a disproportionate distribution in the invertebrates, with glycine usually being one of the major components. Proline, glutamic acid, aspartic acid, and alanine also frequently occur in large amounts (see review of Awapara, 1962). *Nephtys* follows this pattern, with glycine accounting for 40% to 60% of all the amino acid nitrogen, resembling the extremely high proportion found in the body-wall of *Arenicola* (Duchâteau-Bosson *et al.*, 1961).

Glycine may have multiple metabolic pathways in polychaetes. In *Nereis*, Ackermann (1955) ascribes more than half the non-volatile non-protein nitrogen of whole body extracts to glycine betaine, and in *Nephtys* glycine probably is involved in the synthesis of the phosphagen, which is either glycocyamine or creatine (Hobson and Rees, 1957). Although the phosphagen, taurocyamine, does not occur in *Nephtys,* the free amine, taurine, does occur as a major tissue component in many invertebrates, including the polychaetes *Audouinia* (= *Cirriformia*) *spirabranchus* (Kurtz and Luck, 1935), *Sabellaria* sp. (Kittredge *et al.,* 1962) and *Arenicola cristata* (Abbott and Awapara, 1960), and since the methods used here would not have detected the presence of taurine it cannot be excluded as a possible constituent in *Nephtys* coelomic fluid.

The distribution of the other free amino acids in *Nephtys* is not unlike that found in other invertebrates which have high levels of the aliphatics linked with the citric acid cycle, namely, alanine, aspartic and glutamic acids (and their amides), and of proline (see review by Awapara, 1962). The presence of considerable arginine may indicate the absence of an active arginase in the coelomic fluid.

In addition to the free amino acids, there is a small amount of peptide nitrogen, around 10–30 mg./100 ml. (at most, no more than 4 mg./100 ml. is due to asparagine and glutamine). The presence of a large number of metabolically active peptides has recently been revealed in *Drosophila* (Simmons and Mitchell, 1962), and the importance of such compounds in other invertebrates remains to be explored.

There is a discrepancy between the total non-peptide, ninhydrin-positive, alcohol-soluble nitrogen and the total amino acid nitrogen found on the chromatograms. Losses from incomplete recovery of the latter are approximately balanced by inclusion of proline (and in some cases asparagine) which does not produce Ruhemann's purple with ninhydrin (McCaldin, 1960). Thus, 10 to 30 mg./100 ml. ninhydrin-positive nitrogen are unaccounted for. In addition to ammonia, substances known to occur in other invertebrates which would react are taurine, discussed above, β-alanine, β-amino-isobutyric acid, histamine and other amines (see Awapara, 1962) and various phosphatides, some of which are known to occur in whole-body extracts of the polychaetes *Lepidonotus*, *Hydroides* and *Neanthes* (= *Nereis*) (Hack *et al.*, 1962).

The remaining non-protein nitrogen, that which reacts with Nessler's reagent but not with ninhydrin and which shows enormous variation, consists of unidentified substances. The occurrence of choline and other betaines is well known in the invertebrates (see Awapara, 1962) and, as mentioned already, glycine betaine is prominent in *Nereis* (Ackermann, 1955). Trimethylamine oxide also is a major component of non-protein nitrogen in the muscle of marine crustaceans (Kermack *et al.,* 1955; Shaw, 1958), although it has not yet been reported in polychaetes. Enzymes for purine degradation are absent from the few marine annelids which have been studied (see Prosser and Brown, 1961, p. 147), and possibly purines accumulate and contribute to the non-protein nitrogen. If so, they are unlikely to be in the form of uric acid, which is absent from the body fluids of *Amphitrite* and *Nereis* and occurs only at low levels in *Chaetopterus* (Wilber, 1948). In this connection, it is of interest that two ultraviolet-absorbing spots regularly occurred on amino acid chromatograms, possibly nucleic acid derivatives, though no identification was attempted.

In the alcohol-precipitable fraction, the α-amino nitrogen derived from protein, 30 to 60 mg./100 ml., probably represents around 0.2 to 0.4 g. protein/100 ml. Hemoglobin is known to occur in *Nephtys* coelomic fluid, and a rough calculation of the amount present can be made from the oxygen-carrying capacity of coelomic fluid, 0.2–0.5 ml./100 ml. (Jones, 1955). Assuming the same oxygen-carrying capacity per gram of hemoglobin in mammals and *Nephtys,* namely 1 volume O_2 capacity equals 0.75 g. hemoglobin (Redfield and Florkin, 1931) there is from 0.15 to 0.38 g. hemoglobin/100 ml. *Nephtys* coelomic fluid. As the above assumption probably exaggerates the efficiency of *Nephtys* hemoglobin, this value may be too low. Hemoglobin thus accounts for well over 50% of the coelomic fluid protein of pooled samples, although it may vary widely between individuals (Jones, 1955).

Alpha-amino nitrogen derived from protein represents only about half the alcohol-precipitable nitrogen. Only a small part of the remainder can be due to basic residues on the proteins, the rest being unidentified. Part of the latter may be due to amino sugars if the alcohol-precipitable carbohydrate is a glycoprotein. The proportion of protein to unidentified substance in the alcohol-precipitable fraction appears to be higher in the breeding season and in brainless worms than in other groups.

Physiological Aspects

Certain points arising from the results have physiological significance for *Nephtys* and require brief comment.

Intertidal populations of *Nephtys hombergi* occur on beaches composed of fairly fine particles (Clark and Haderlie, 1960). The worms live in unconsolidated burrows, and during the long periods of tidal exposure the oxygen supply is insufficient to maintain respiration (Jones, 1955). Moreover, *Nephtys* accumulates lactic acid when kept at low oxygen tensions in the laboratory (unpublished observations), indicating active glycolysis. Thus, the high buffering capacity of the coelomic fluid is to be expected.

Although starvation causes a fall in free sugars in the coelomic fluid of non-breeding *Nephtys,* it is remarkable that the decrease in total carbohydrate is proportionately much less because of the increase in the alcohol-insoluble fraction. The normal high levels of sugars in *Nephtys* coelomic fluid and the fact that after several days' starvation they are still higher than in other polychaetes examined show that *Nephtys* has considerable reserves. Although nothing is known of the feeding behaviour in this species, it is physiologically equipped for infrequent feeding and long periods of inanition. This is further confirmed by the fact that secondary sources of energy, such as amino acids, are not depleted during starvation.

The maintenance of blood sugars at nearly normal levels, even when starved, by worms during the breeding season and by brainless worms, in which sexual maturation has been provoked, may indicate a mobilization of sugars into the coelomic fluid during maturation for utilization by the gametes. Dales (1957) has suggested that body fat and glycogen are probably transferred to gametes during maturation in other polychaetes, and this may also occur in *Nephtys*. Whether the alcohol-precipitable carbohydrate is involved in this process is not clear, although it shows remarkable fluctuations and appears to increase in both starved and breeding animals. Lack of digestion by β-amylase indicates that this is not glycogen. If this high-molecular-weight carbohydrate is a glycoprotein, it might contribute to the free sugars. A lysozyme is present in *Nephtys hombergi* (Jollès *et al.*, 1958; Jollès and Zuili, 1960) which could bring about the initial degradation. On the other hand, in crabs, the blood glycoprotein, although composed of glucose and glucosamine, is not in direct equilibrium with glucose or maltose (Meenakshi and Scheer, 1961).

During gamete resorption there is likely to be a sharp reduction in utilization of sugars, which may be the explanation of the high levels found in those worms collected shortly after the end of the breeding season (Batches II and III). If the rate of production and release of precursor substances has declined, this would explain why post-breeding animals are unable to maintain sugar levels during starvation. Only on brain removal is there a renewed mobilization of carbohydrate. The slight decrease in carbohydrate levels during regeneration in non-breeding worms suggests an increased utilization of carbohydrates. Glycogenolysis in and near regenerating regions of the earthworm has been reported by O'Brien (1957). The present data, however, need further amplification before any conclusions can be drawn in this respect.

The significance of high concentrations of free amino acids in *Nephtys* coelomic fluid remains obscure, although they may relate to osmoregulation. It has been shown that various polychaetes are able to take up free amino acids from the external medium (Stephens and Schinske, 1961; Stephens, 1962a, 1962b), including *Nereis limnicola* and *N. succinea,* which are both euryhaline but to different degrees (Stephens, 1963). In these two species, the rate of glycine uptake is inversely proportional to the external salinity, the rate falling off abruptly when the animals start to regulate internal chloride concentration. Marine forms generally have far higher levels of amino acids in their tissues than do their fresh water or terrestrial relatives (see Awapara, 1962), and changes in the intracellular free amino acid levels occur both in poikilosmotic and in osmoregulating polychaetes (Jeuniaux *et al.,* 1961a, 1961b; Duchâteau-Bosson *et al.,* 1961). However, only in insects has such a high amino-acidemia as occurs in *Nephtys* previously been reported, where, in the larvae of aquatic species, it fluctuates inversely with alterations in the external salinity (see Florkin, 1963). In this regard, *Nephtys hombergi,* the species used here, is more euryhaline than most other species of this genus (Clark and Haderlie, 1960), and a comparative study of amino acid levels in the coelomic fluid of other species would thus be of interest.

Aside from osmoregulation, physiological changes in the amount and distribution of free amino acids in invertebrates are almost unexplored. One possible function of amino acids may be to form complexes with heavy-metal ions present in sea water; this has been suggested by Tyler and Rothschild (1951) as an explanation of their role in prolonging the motility of shed sea urchin sperm. In the present study there is no consistent change either in total levels or in amounts of particular amino acids under any of the experimental conditions, and certainly no regular decrease in free amino acids on starvation, as occurs with the free sugars in *Nephtys,* although there may be a tendency for amino acids to be higher in breeding animals and in those with their brains removed.

Biochemical changes correlated with regeneration were not forthcoming except for the increase in pH of 0.2 to 0.3 unit which was repeatedly seen in the coelomic fluid of three-day regenerating worms. This does not appear to be due merely to a short period of starvation, and may result from temporary exposure to sea water after caudal amputation. At 10 days, starved and regenerating worms showed the same coelomic fluid pH values.

On the other hand, it is clear from the changes in both carbohydrate and nitrogen metabolism that brain removal produces a biochemical picture resembling that of breeding worms. When the carbohydrate chemistry is better understood it will no doubt prove rewarding to investigate changes in the activities of the carbohydrases concerned; and the identification of the non-amino, alcohol-soluble nitrogen fraction, which is little affected by starvation but is increased during the breeding season and after brain removal, may provide an explanation of its role in maturation.

The author is grateful to Dr. R. B. Clark for performing the brain extirpations and for helpful discussion of the biological aspects of this work, and to Dr. B. F. Folkes for useful discussion of some of the biochemical problems and for reading the manuscript. Thanks are also due to Drs. A. P. Sims and D. K. Lewis for suggesting the quantitative ninhydrin method for amino acid estimations.

SUMMARY

1. The coelomic fluid of *Nephtys hombergi* was examined with respect to its pH, buffering capacity, and carbohydrate and nitrogen distributions.

2. The pH of normal worms lies between 6.4 and 6.6, and the buffering capacity is 8 meq/l./pH unit between pH 4.5 and 7.5.

3. The carbohydrates are distributed between a small alcohol-insoluble fraction (not glycogen) and an alcohol-soluble fraction, shown by chromatography to consist of 50–70 mg./100 ml., each, of glucose and maltose.

4. The alcohol-insoluble nitrogenous compounds range from 70–150 mg./100 ml. Only about half is due to α-amino groups from protein and the rest is unidentified.

5. The alcohol-insoluble nitrogen lies between 180–260 mg./100 ml. Of this, about 90 mg. are due to α-amino nitrogen and ammonia, about 10 mg. to peptides, and the remainder is unknown. Quantitative amino acid chromatography gave total amino acid levels at between 40–60 mg./100 ml., about half of which is glycine. Alanine and proline are also prominent.

6. Starvation for 10 days has no consistent effect on nitrogen distribution, but it causes a fall in free sugars and an increase in alcohol-precipitable carbohydrates, except in breeding worms.

7. Breeding worms are able to maintain a high level of free sugars despite starvation and also show an increase in the unidentified alcohol-soluble nitrogenous fraction. A similar picture is brought about in non-breeding worms by extirpation of the supraoesophageal ganglion, an operation which also induces precocious maturation.

8. Caudal amputation of segments has little effect on coelomic fluid chemistry except temporarily to raise the pH by 0.2 to 0.3 unit around the third day of regeneration. This may be the result of exposure of the coelom to sea water.

LITERATURE CITED

Abbott, W., and J. Awapara, 1960. Sulfur metabolism in the lugworm, *Arenicola cristata* Stimpson. *Biol. Bull.*, **119**: 357–370.

Ackermann, D., 1955. Über das Vorkommen von Glycocyamin, Cholin, Lysin, Leucin und α-Alanin in dem Meereswurm, *Nereis virens*. *Zeitschr. physiol. Chem.*, **299**: 186–190.

Awapara, J., 1962. Free amino acids in invertebrates: a comparative study of their distribution and metabolism. *In:* Amino Acid Pools, J. T. Holden, Ed., Elsevier, New York, pp. 158–175.

Barcroft, J., 1934. Features of the Architecture of Physiological Function. University Press, Cambridge.

Britton, H. G., 1962. Some non-reducing carbohydrates in animal tissues and fluids. *Biochem. J.*, **85**: 402–407.

Camien, M. N., H. Sarlet, G. Duchâteau and M. Florkin, 1951. Non-protein amino acids in muscle and blood of marine and fresh-water Crustacea. *J. Biol. Chem.*, **193**: 881–885.

Chinard, F. P., 1952. Photometric estimation of proline and ornithine. *J. Biol. Chem.*, **199**: 91–95.

Clark, M. E., and R. B. Clark, 1962. Growth and regeneration in *Nephtys*. *Zool. Jahrb., Abt. Physiol.*, **70**: 24–90.

Clark, R. B., and M. E. Clark, 1959. Role of the supraoesophageal ganglion during the early stages of caudal regeneration in some errant polychaetes. *Nature*, **183**: 1834–1835.

Clark, R. B., and E. C. Haderlie, 1960. The distribution of *Nephtys cirrosa* and *N. hombergi* on the southwestern coasts of England and Wales. *J. Anim. Ecol.*, **29**: 117–147.

Clark, R. B., and R. J. G. Ruston, 1963. Time of release and action of a hormone influencing regeneration in the polychaete *Nereis diversicolor*. *Gen. Comp. Endocrinol.*, **3**: 542–553.

Clark, R. B., M. E. Clark and R. J. G. Ruston, 1962. The endocrinology of regeneration in some errant polychaetes. *In:* Proc. III Internat. Conf. Neurosecretion, H. Heller and R. B. Clark, Eds. *Mem. Soc. Endocrinol.*, **12**: 275–286.

Cole, W. H., 1940. The composition of fluids and sera of some marine animals and of the sea water in which they live. *J. Gen. Physiol.*, **23**: 575–584.

Dales, R. P., 1957. Preliminary observations on the role of the coelomic cells in food storage and transport in certain polychaetes. *J. Mar. Biol. Assoc.*, **36**: 91–110.

Dent, C. E., 1948. A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-'collidine' filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.*, **43**: 169–180.

Duchâteau, G., and M. Florkin, 1958. A survey of amino-acidemias with special reference to the high concentration of free amino in insect hemolymph. *Arch. Internat. Physiol. Biochim.*, **66**: 573–592.

Duchâteau-Bosson, G., C. Jeuniaux and M. Florkin, 1961. Rôle de la variation de la composante amino-acide intracellulaire dans l'euryhalinité d'*Arenicola marina*. *Arch. Internat. Physiol. Biochim.*, **69**: 30–35.

Durchon, M., and M. Lafon, 1951. Quelques donnés biochimiques sur les Annélides. *Ann. Sci. Nat. Zool., Ser. xi*, **13**: 427–452.

Fairbairn, D., 1958. Trehalose and glucose in helminths and other invertebrates. *Canad. J. Zool.*, **36**: 787–795.

Florkin, M., 1934. La fonction respiratoire du "millieu intérieur" dans la série animale. *Ann. Physiol. Physicochim. Biol.*, **10**: 599–694.

Florkin, M., 1963. L'évolution biochimique et la radiation physiologique des systèmes biochimiques chez les animaux. *In:* Proc. V Internat. Cong. Biochem., vol. III, Evolutionary Biochemistry, A. I. Oparin, Ed., Macmillan, New York, pp. 250–270.

Fong, J., F. L. Schaffer and P. L. Kirk, 1953. The ultramicrodetermination of glycogen in liver. A comparison of the anthrone and reducing-sugar methods. *Arch. Biochem. Biophys.*, **45**: 319–326.

Giordano, M. F., H. A. Harper and F. D. Filice, 1950. The amino acids of the blood of *Urechis caupo* (Echiurida). *Wasmann J. Biol.*, **8**: 1–7.

Hack, M. H., A. E. Gussin and M. E. Lowe, 1962. Comparative lipid biochemistry—I. Phosphatides of invertebrates (Porifera to Chordata). *Comp. Biochem. Physiol.*, **5**: 217–221.

Harris, C. K., E. Tigane and C. S. Hanes, 1961. Quantitative chromatographic methods. Part 7. Isolation of amino acids from serum and other fluids. *Canad. J. Biochem. Physiol.*, **39**: 439–451.

Heimpel, A. M., 1955. The pH in the gut and blood of the larch sawfly, *Pristiphora erichsonii* (HTG), and other insects with reference to the pathogenicity of *Bacillus cereus* Fr. and Fr. *Canad. J. Zool.*, **33**: 99–106.

Heimpel, A. M., 1956. Further observations on the pH in the gut and the blood of Canadian forest insects. *Canad. J. Zool.*, **34**: 210–212.

Hobson, G. E., and K. R. Rees, 1957. The annelid phosphokinases. *Biochem. J.*, **65**: 305–307.

Jeuniaux, C., G. Duchâteau-Bosson and M. Florkin, 1961a. Free amino acids in the intracellular osmoregulation of euryhaline marine worms. *Biochem. J.*, **79**: 24–58.

Jeauniaux, C., G. Duchâteau-Bosson and M. Florkin, 1961b. Variation of the amino acid composition of tissues, and euryhalinity between *Perinereis cultrifera* Gr. and *Nereis diversicolor* (O. F. Müller). *J. Biochem. (Tokyo)*, **49**: 527–531.

Jollès, P., J. Jollès-Thaureaux and C. Fromageot, 1958. Recherche de lysozymes chez quelques Invertébrés. Lysozyme de *Nephthys hombergi. C. R. Soc. Biol.*, **151**: 1368–1369.

Jollès, P., and S. Zuili, 1960. Purification et étude de nouveaux lysozymes; extraits du poumon de poule et de *Nephthys hombergi. Biochim. Biophys. Acta*, **39**: 212–217.

Jones, B. M., and I. Cunningham, 1961. Growth by cell division in insect tissue culture. *Exp. Cell Res.*, **23**: 386–401.

Jones, J. D., 1955. Observations on the respiratory physiology and on the haemoglobin of the polychaete genus *Nephthys*, with special reference to *N. hombergii* (Aud. et M.-Edw.). *J. Exp. Biol.*, **32**: 110–125.

Kermack, W. O., H. Lees and J. D. Wood, 1955. Some non-protein constituents of lobster tissues. *Biochem. J.*, **60**: 424–428.

Kittredge, J. S., D. G. Simonsen, E. Roberts and B. Jelinek, 1962. Free amino acids of marine invertebrates. *In:* Amino Acid Pools, J. T. Holden, Ed., Elsevier, New York, pp. 176–186.

Kleinholz, L. H., and B. C. Little, 1949. Studies in the regulation of blood-sugar concentration in crustaceans I. Normal values and experimental hyperglycemia in *Libinia emarginata. Biol. Bull.*, **96**: 218–227.

Kurtz, A. C., and J. M. Luck, 1935. Studies on annelid muscle. I. Taurine in *Audouinia spirabranchus*, Moore. *J. Biol. Chem.*, **111**: 577–584.

McCaldin, D. J., 1960. Chemistry of ninhydrin. *Chem. Rev.*, **60**: 39–51.

Meenakshi, V. R., and B. T. Scheer, 1961. Metabolism of glucose in the crabs *Cancer magister* and *Hemigrapsus nudus. Comp. Biochem. Physiol.*, **3**: 30–41.

Mitchell, H. K., and J. R. Simmons, 1962. Amino acids and derivatives in *Drosophila. In:* Amino Acid Pools, J. T. Holden, Ed., Elsevier, New York, pp. 136–146.

Mukherjee, S., and H. C. Srivastava, 1952. Improved spray reagent for detection of sugars. *Nature*, **169**: 330.

O'Brien, B. R. A., 1957. Tissue metabolism during posterior regeneration in the earthworm. *Aust. J. Exp. Biol. and Med. Sci.*, **35**: 373–380.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology (2nd edition). Saunders, Philadelphia.

Redfield, A. C., and M. Florkin, 1931. The respiratory function of the blood of *Urechis caupo. Biol. Bull.*, **61**: 185–210.

Redfield, A. C., and E. N. Ingalls, 1932. The effect of salts and hydrogen-ion concentration upon the oxygen dissociation constant of the hemocyanin of *Busycon canaliculatum*. *J. Cell. Comp. Physiol.*, **1**: 253–275.

Redfield, A. C., G. Humphreys and E. N. Ingalls, 1929. Hemocyanin buffer action in *Limulus* blood. *J. Biol. Chem.*, **82**: 759–773.

Redfield, R. R., 1953. Two-dimensional paper chromatographic systems with high resolving power for amino acids. *Biochim. Biophys. Acta*, **10**: 344–345.

Scheer, B. T., and M. A. R. Scheer, 1951. The hormonal control of metabolism in crustaceans —I. Blood sugar in spiney lobsters. *Physiol. Comp. Oecologia*, **2**: 198–209.

Scully, U., 1964. Factors influencing the secretion of regeneration-promoting hormone in *Nereis diversicolor*. *Gen. Comp. Endocrinol.*, **4**: (in press).

Seifter, S., S. Dayton, B. Novic and E. Muntwyler, 1950. The estimation of glycogen with the anthrone reagent. *Arch. Biochem.*, **25**: 191–200.

Seton, E., and C. G. Wilber, 1949. Glucose metabolism in marine annelids. *Biol. Bull.*, **97**: 246.

Shaw, J., 1958. Osmoregulation in the muscle fibres of *Carcinus maenas*. *J. Exp. Biol.*, **35**: 920–929.

Simmons, J. R., and H. K. Mitchell, 1962. Metabolism of peptides in *Drosophila*. *In:* Amino Acid Pools, J. T. Holden, Ed., Elsevier, New York, pp. 147–155.

Somogyi, M., 1945. Determination of blood sugar. *J. Biol. Chem.*, **160**: 69–73.

Stephens, G. C., 1962a. Amino acids in the economy of the bamboo worm, *Clymenella torquata*. *Biol. Bull.*, **123**: 512.

Stephens, G. C., 1962b. Uptake of amino acids by the bamboo worm, *Clymenella torquata*. *Biol. Bull.*, **123**: 512.

Stephens, G. C., 1963. The influence of salinity on the uptake of glycine by euryhaline annelids. *In:* Proc. XVI Internat. Cong. Zool., vol. II, p. 41.

Stephens, G. C., and R. A. Schinske, 1961. Uptake of amino acids by marine invertebrates. *Limnol. and Oceanog.*, **6**: 175–181.

Stevens, T. M., 1961. Free amino acids in the hemolymph of the American cockroach, *Periplaneta americana* L. *Comp. Biochem. Physiol.*, **3**: 304–309.

Stevens, T. M., C. E. Howard and R. W. Schlesinger, 1961. Free amino acids in sera of the marine invertebrates, *Cancer irroratus*, *Limulus polyphemus*, and *Homarus americanus*. *Comp. Biochem. Physiol.*, **3**: 310–314.

Toennies, G., and J. J. Kolb, 1951. Techniques and reagents for paper chromatography. *Anal. Chem.*, **23**: 823–826.

Tyler, A., and Lord Rothschild, 1951. Metabolism of sea-urchin spermatozoa and induced anaerobic mobility in solutions of amino acids. *Proc. Soc. Exp. Biol. Med.*, **76**: 52–58.

Weed, L. L., and T. A. Courtenay, 1954. A new nucleotide and a new polynucleotide from bacteriophage nucleic acid. *J. Biol. Chem.*, **206**: 735–740.

Wellington, E. F., 1952. An ultra-micro method for quantitative determination of amino acids. *Canad. J. Chem.*, **30**: 581–591.

Wellington, E. F., 1953. Effect of relative humidity on the reaction of ninhydrin with amino acids on paper chromatograms. *Canad. J. Chem.*, **31**: 484–487.

Wilber, C. G., 1948. Uric acid in body fluids of marine invertebrate animals. *J. Cell. Comp. Physiol.*, **31**: 107–109.

Woolf, L. I., 1953. Use of ion-exchange resins in paper chromatography of sugars. *Nature*, **171**: 841.

Wyatt, G. R., 1961. The biochemistry of insect hemolymph. *Ann. Rev. Entomol.* **6**: 75–102.

Wyatt, G. R., T. C. Loughheed and S. S. Wyatt, 1956. The chemistry of insect hemolymph. Organic components of the hemolymph of the silkworm, *Bombyx mori*, and two other species. *J. Gen. Physiol.*, **39**: 853–868.

THE INFLUENCE OF LIGHT ON CELL AGGREGATION IN POLYSPHONDYLIUM PALLIDUM

ARNOLD J. KAHN [1, 2]

Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

The effect of light on aggregation in the cellular slime molds has been known since Potts (1902) observed that *Dictyostelium mucoroides* formed more numerous and smaller aggregates when incubated in the light than in the dark. Harper (1932) and Raper (1940) made similar observations on *Polysphondylium violaceum* and *D. discoideum,* respectively, while Konijn and Raper (unpublished data) found comparable phenomena to exist in *P. pallidum* and *D. purpureum.* The influence of light on cell aggregation is not limited to the more complex species; for example, Olive and Stoianovitch (1960) found that in the absence of light the myxamoebae of *Acrasis rosea* fail to develop beyond the vegetative stage and generally undergo encystment. This observation has been extended by Reinhardt (personal communication) to show that it is the relationship between light *and* dark, rather than exposure to light, that is important in the development of this organism.

In the present study, the effect of light on cell aggregation and aggregation density (*i.e.*, the number of aggregates per unit area) in *Polysphondylium pallidum* has been examined in some detail with particular attention to the nature of the light response. The process of aggregation has been observed, *in situ*, under natural conditions of growth, and as it is affected by various modifications in the cultural environment. Based on these observations and experiments, and those of other authors, a hypothesis is presented which may account for the effect of light and may help to explain certain features of aggregation such as the time of onset and the number of centers per unit area.

MATERIALS AND METHODS

The species investigated was *Polysphondylium pallidum* Olive, strain WS-320. The stock cultures were maintained on dilute hay-infusion agar (Raper, 1951) in association with *Escherichia coli*, strain B/r. For the majority of experiments, the myxamoebae were grown on a suspension of *E. coli* cells in *M*/60 phosphate buffer, following Hohl's and Raper's (1963) modification of Gerisch's technique (1960), and harvested in the stationary phase. The cells were washed essentially free of bacteria by differential centrifugation, and were resuspended in Bonner's salt solution (Bonner, 1947) to a concentration of 2×10^6 to 1×10^7 cells/ml. and dispensed in 0.01-ml. drops on an agar substrate consisting of 1.5% Bacto purified agar in Bonner's salt solution. It is most important that the agar be prepared and

---

[1] Postdoctoral Fellow, Department of Bacteriology, University of Wisconsin, Madison.

[2] Present address: Department of Zoology, Syracuse University, Syracuse 10, New York.

poured into Petri dishes 40 to 48 hours prior to use, to allow the evaporation of the excess liquid. If this is done, the liquid from the drop will enter the agar within about an hour, leaving a circle of uniformly distributed myxamoebae. Such circles cover an area, on the average, of from 10 to 12 mm.^{2}, thereby yielding cell densities on the substrate of from 1.7×10^3 to 1×10^4 cells per mm^2.

The plates were incubated at 20° C. and scored for the number of centers of aggregation per drop after 22 to 26 hours. Illumination when required was provided by a single 15-watt daylight fluorescent bulb (maximal emission between 430 and 615 mμ) placed at a distance of 22–25 cm. from the cultures and giving an intensity of about 75 foot-candles at the level of the agar. In other experiments conducted under either "dark" conditions or requiring dark-grown myxamoebae, all procedures (centrifugation, dispensation of aliquots, *etc.*) were carried out in a room dimly lit with a Kodak safelight equipped with a red Wratten filter. Darkness during incubation was maintained by keeping the plates in light-tight Petri dish canisters.

For studies of cell growth and aggregation on the same plate, Petri plates containing a nutrient gel (0.3% lactose, 0.15% peptone, and 1.5% agar, phosphate buffered to pH 6.0–6.2) were spread with a suspension of bacteria and inoculated in the center with spores of *P. pallidum*. Incubation was at 20° C.

RESULTS

In the first series of experiments, myxamoebae were grown in the dark in liquid culture, processed (harvested, washed, *etc.*), and exposed to light for various lengths of time following deposition on agar, and then returned to the dark. The results of two such experiments are shown in Figure 1.

The outstanding feature of this graph is the increase shown in the number of centers formed by the myxamoebae with increasing exposure to light. For the two experiments in Figure 1, this amounted to a 6-fold (upper curve) and a 12-fold (lower curve) difference in the number of centers for cultures under continuous illumination, while in still another experiment a 31-fold difference was observed. Not only is there an increase in the number of centers, but the interval between the time of deposition of the drops and the onset of aggregation is shortened by exposure to light. This "hastening" of aggregation by light has been examined in some detail by Raper (1940), Shaffer (1958) and Konijn and Raper (unpublished data). Clearly, then, light can play a major role not only in determining the number of centers that will form in a population of myxamboebae, but also in hastening the time at which they appear. When is light most effective? From these experiments and some that follow, it appears that light becomes effective shortly after the deposition of the drops, generally within the first two to three hours of incubation. During this period, even a comparatively short exposure to light is quite effective in increasing the number of centers.

The degree of sensitivity of the light response mechanism is indicated by the fact that one minute of light appeared to be almost as effective in inducing center formation as were 120 minutes (Fig. 2). In other words, under the conditions of these experiments, a small amount of light at the "right time" was particularly effective in increasing the number of centers. Note also the difference in the level

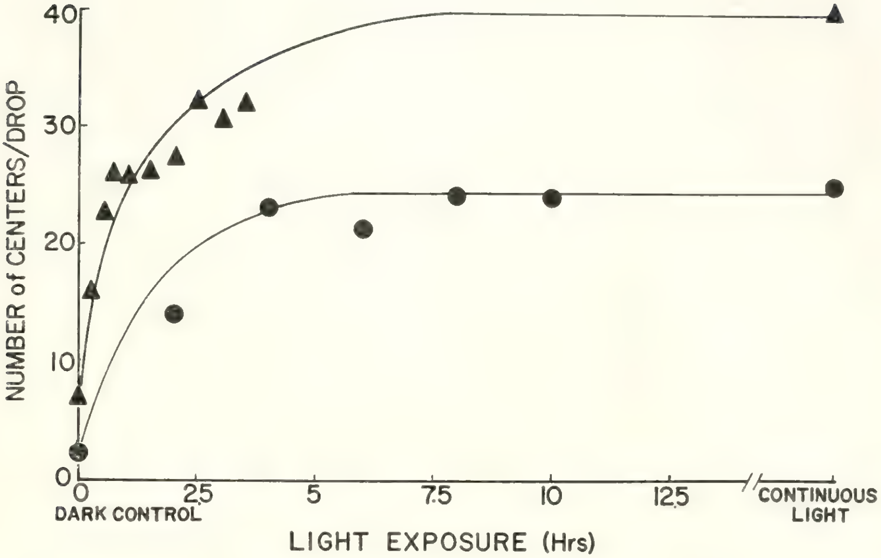


FIGURE 1. The number of aggregation centers formed per drop is plotted as a function of exposure to light. The myxamoebae were grown in the dark, deposited on agar, and exposed to light; they were then returned to the dark following various time intervals. Counts of the number of centers were made after 24 to 26 hours of incubation. Each point represents the mean of 15 to 20 values. The two curves represent different experiments.

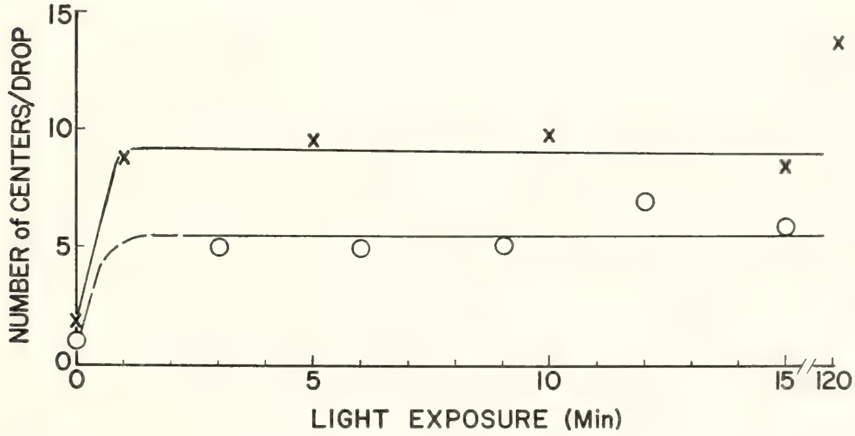


FIGURE 2. The effect of varying light exposure on aggregation. Dark-grown myxamoebae were exposed to light for from 3 to 15 minutes in one case (O) and from 1 to 120 minutes in the other (X); they were then returned to dark. In the latter experiment (X), the light exposure was made after dark incubation of two hours; in the former (O), the exposure followed four hours of dark incubation. Note that the values after four hours are less than those after two hours. Each point represents the mean value of 15 to 20 replicate drops.

of response between the two experiments illustrated in Figure 2. In the upper curve, the myxamoebae were exposed to light after two hours of dark incubation, in the lower, after four hours. This difference in responsiveness would suggest a loss in light sensitivity during prolonged dark incubation. To check this possibility, dark-grown myxamoebae were exposed to 15 minutes of light at various times during incubation and then returned to the dark. The results of three experiments are shown in Figure 3.

Two features are apparent in these experiments. One, populations of myxamoebae which receive light form a greater number of centers of aggregation than those maintained in darkness. Two, the effectiveness of light diminishes during incubation, *i.e.*, cultures that receive light early during incubation form a greater number of centers than similar cultures that receive the same amount of light later. Indeed, cells which are not incorporated into aggregates during the period of maximum sensitivity may never aggregate, even when exposed to light for much longer periods. These unincorporated cells form microcysts, *i.e.*, encysted individual myxamoebae. (For a discussion of the encystment stages of cellular slime molds see Blaskovics and Raper, 1957.)

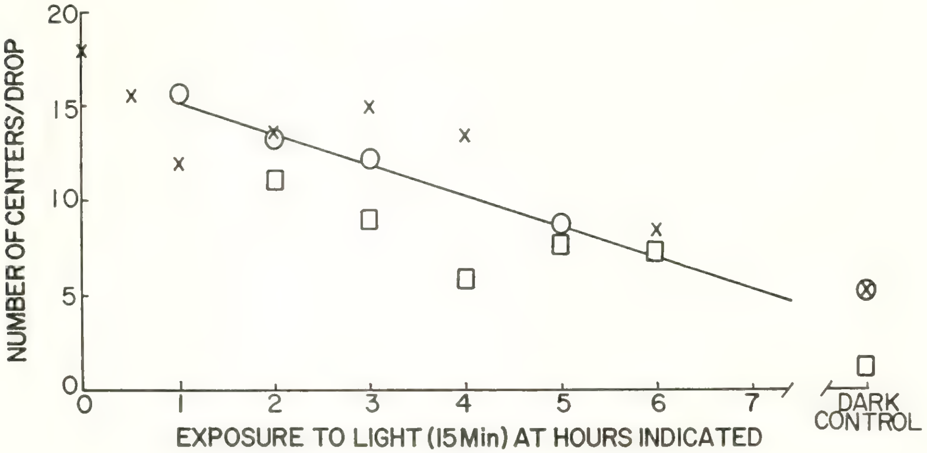


FIGURE 3. The effect of short-term light exposure on aggregation. Dark-grown myxamoebae were exposed to light for single 15-minute periods at various times during incubation and then returned to the dark. Scoring for the number of centers took place 24 hours after the deposition of the drops. Each point represents the mean of 15 to 20 values. The symbols X, O, and □ represent three different experiments.

Studies of myxamoeba populations grown in situ

Up to this point, we have examined the influence of light on isolated populations of pregrown myxamoebae deposited on non-nutrient agar. It seemed of interest, therefore, to know the response of myxamoebae under more routine culture conditions. For such observations agar plates containing 0.3% lactose-0.1% peptone as nutrient were spread with a suspension of *E. coli* B/r and inoculated in the center

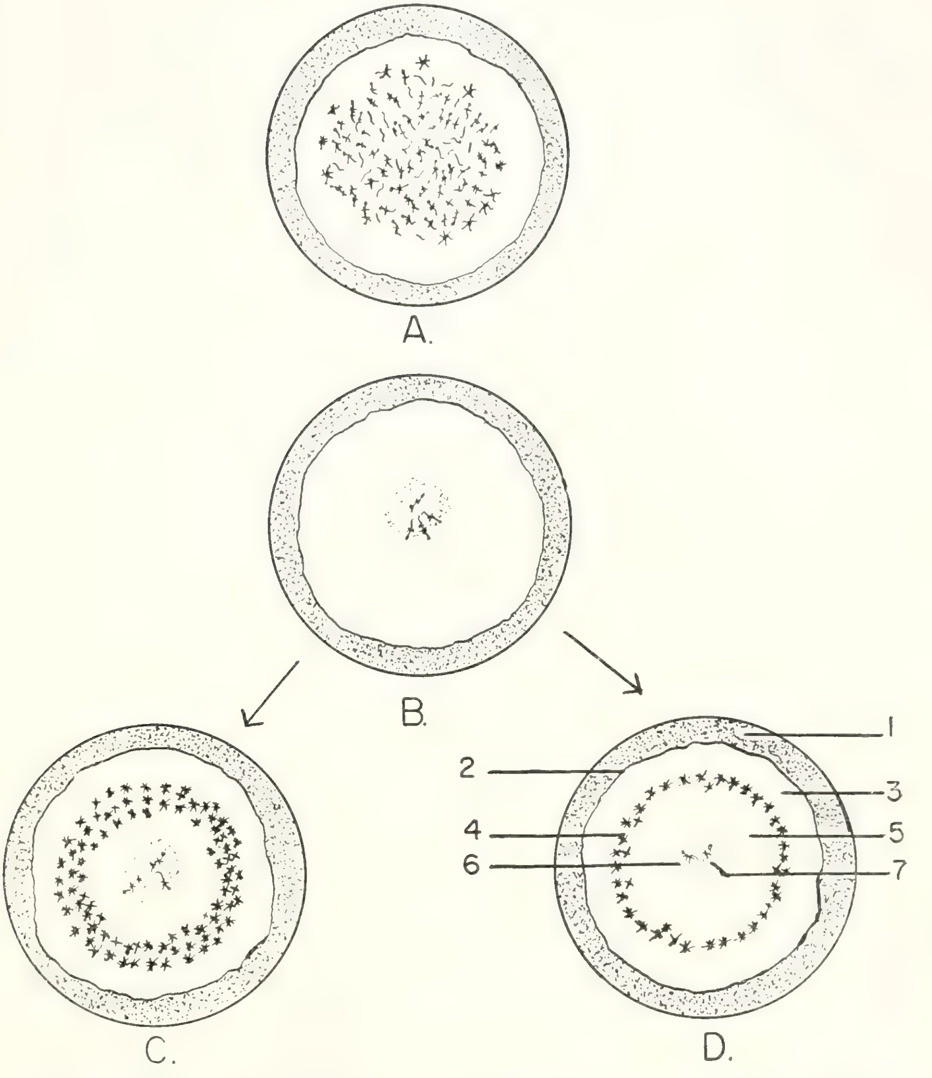


FIGURE 4. Diagram illustrating the behavior of cultures grown *in situ* under conditions of continuous light (A), continuous darkness (B), and after exposure of dark-grown cultures to light (C), and to charcoal (D). Note the differences in the levels of development in the light- and charcoal-treated cultures: (1) unconsumed bacteria, (2) feeding front (bacteriophagic activity), (3) "young" post-feeding myxamoebae, (4) aggregation, (5) "old" post-feeding myxamoebae, (6) microcysts, and (7) limited sorocarps at or near the point of inoculation which may or may not be produced.

with spores of *P. pallidum*. When incubated in the light such plates show a progression of growth and subsequent morphogenesis from the point of inoculation to the periphery of the agar surface (Fig. 4, A). Plates, similarly prepared but inoculated in the dark, sometimes show the presence of sorocarps at or near the point of inoculation but fail to display any further sign of fructification by the remaining myxamoebae (Fig. 4, B). The fact that some sorocarps do develop is of some interest. For the moment, suffice it to say that this apparently does not result from some center-inducing factor carried by the spores which could initiate aggregation.

If the plates prepared in the manner described above are incubated in the dark for 5 or 6 days and then exposed to light, center formation will occur only within a limited circular zone. The remainder of the plate shows, from the point of inoculation to the margin, areas in which the following developmental stages may be seen: sorocarps (usually less than 10), microcysts, "old" myxamoebae, aggregations, or sorocarps, "young" post-feeding (interphase) myxamoebae, feeding myxamoebae, and bacteria (Fig. 4, D). By marking the daily increment of growth as the colony expands, and correlating "age" with the time of light exposure, it has been determined that the myxamoebae in the zone of aggregation or culmination are two to three days old. Furthermore, a much greater exposure to light (hours instead of minutes) is required to induce aggregation in these cells than in the isolated populations previously discussed. Older cells, under these conditions, show no signs of activity. On the basis of this difference in behavior, it would appear that, as in isolated populations, the ability to aggregate as a response to light is limited in time, *i.e.,* to rather "young" myxamoebae.

The same type of response occurs if similar cultures incubated in continuous darkness are exposed to vegetable charcoal (*cf.* Bonner and Hoffman, 1963). That is, a band or zone of sorocarp formation and cell aggregation appears near the periphery of the expanding colony. Moreover, this zone often appears more quickly, is of greater width (*i.e.,* encompasses still "older" areas of myxamoebae), and shows a greater density of aggregation centers than in comparable cultures exposed to light (Fig. 4, C). But more than this, the myxamoebae within the broader zone respond at about the *same* time. This "synchrony" is also evident in light-induced cultures, but is less striking because the responding myxamoebae are more nearly of the same age. In charcoal-treated cultures, the unaggregated myxamoebae within a zone may differ in chronological age by a matter of *four* to *five* days. It is as if the myxamoebae, upon reaching a certain stage of development, underwent a developmental "freeze" which kept them primed for aggregation for several days. This is in contrast to (1) isolated populations which show, over a short period of time, a decline in the ability to aggregate as a response to light (Fig. 3), and to (2) cultures grown *in situ* in the light which show continuous morphogenetic activity in the zone between the feeding front and the mature sorocarps. In the latter instance, there is a linear sequence in the stages of development from aggregation to culmination.

If myxamoebae four to five days old are still capable of aggregating, it is rather surprising to find areas of a culture which are free of aggregates. When viewed under the microscope, the myxamoebae in these areas appear much as they do in other parts of the culture, with none of the features of dead or dying cells such as a high degree of vacuolation and Brownian movement. What prevents these cells from completing the life cycle? A partial answer to this question is given in the results of some preliminary experiments. For example, the excision and transfer of a center of aggregation to an area populated by these cells elicits, at best, a very weak chemotactic response. Assuming that the transplants are active producers of an attractant (acrasin), this observation would indicate a defect in the chemotactic response mechanism. On the other hand, if such cells are collected, resuspended and deposited as isolated populations on a fresh non-nutrient substrate, they respond by forming pseudoplasmodia and sorocarps of perfectly normal appearance. This observation, taken together with the fact that charcoal is effective in stimulating center formation, suggests that there is something present in the environment and removable by charcoal which is responsible for inhibiting aggregation.

Effect of certain agents on aggregation density

It is evident from the material presented above that in *P. pallidum,* light does have a very real and dramatic effect upon the initiation of cell aggregation and upon the density of such aggregations. What is much less evident is how light acts to achieve this effect. A hypothesis stating simply that light is required for aggregation

TABLE I

The effect of various agents on aggregation density. The first value in each column represents the mean number of centers per drop; the number of drops averaged is in parenthesis

Agent	Control	Experimental
1. Myxamoebae grown in the *dark*, harvested, washed and		
a) Incubated in the dark:		
Heat*	1.7 (40)	1.3 (40)
Light Cells$^+$	1.5 (60)	1.5 (37)
CO_2‡	2.0 (20)	1.5 (20)
KOH**	1.0 (40)	1.1 (40)
Charcoal^{++}	1.5 (40)	5.8 (40)
Mineral Oil‡‡	1.3 (40)	18.3 (40)
b) Incubated in the light:		
Heat	—	—
Light Cells	21.0 (19)	21.7 (20)
CO_2	23.5 (20)	13.5 (20)
KOH	23.5 (20)	23.0 (20)
Charcoal	16.7 (56)	26.2 (60)
Mineral Oil	15.8 (56)	43.2 (49)
2. Myxamoebae grown in the *light*, harvested, washed and		
a) Incubated in the dark—1.1 (37)		
b) Incubated in the light—23.4 (38)		

* Dark-grown myxamoebae were exposed for 15 and 30 minutes to a temperature of 33° C.

$^+$ Dark-grown myxamoebae were combined in mixed populations with cells preincubated in the light for one or two hours, *i.e.*, sufficient to elicit aggregation. The proportions were approximately 20% "light"-grown to 80% "dark"-grown cells.

‡CO_2 was provided by placing pieces of dry ice, about one cubic centimeter in volume, in the lids of inverted cultures.

** Large well slides containing several milliliters of KOH solution (5% and 10%) were placed in the lids of inverted cultures.

$^{++}$ The charcoal was placed in aluminum cups which were either embedded into the agar to form a receptable or placed in the lids of inverted cultures.

‡‡ After the excess liquid from the drop was absorbed into the agar, the myxamoebae were covered with a layer of mineral oil.

is untenable since aggregation can and does occur in its absence. There are a number of possible hypotheses which could account for the effect of light on aggregation. Among these are (1) the destruction, or inactivation, of acrasin, and (2) the alteration in the response of myxamoebae to chemotactic stimuli. The notion most readily accessible to experimentation, and that suggested, in part, by the preceding experiments, is that light operates by changing in some way the relationship between the process of aggregation and an aggregation (or center) suppressing factor produced by the cells and present in the environment. For example, light might limit the production of or, perhaps, destroy such a factor, thereby permitting aggregation to occur. This view is consistent with the recent proposals of Bonner and Dodd (1962) and Bonner and Hoffman (1963). To test this notion, and some others, certain experiments were performed, the results of which are presented in Table I. Because there is some variation in the behavior of myxamoebae from one experiment to another, values are compared only within the same experiment or group of experiments. In evaluating these results, it is assumed that if a suppressor or "spacing substance" is involved, then its removal should lead to an increase in the number of centers.

To summarize these results on a point-by-point basis:

Heat appears not to be an effective replacement for light. This observation is of some importance since it tends to eliminate from these experiments one of the factors, a rise in temperature, reported by Raper (1940) as important in increasing the density of aggregation in *Dictyostelium discoideum.*

Myxamoebae illuminated sufficiently to initiate aggregation do not impart this ability to dark-incubated cells and appear themselves to be limited by the associated "dark cells" from forming centers. This observation, in and of itself, is a rather strong argument against a hypothesis based simply on the light-induction of centers.

While CO_2 had little effect on dark-incubated cultures, it sharply reduced the number of aggregates formed in the light. This is in agreement with the observations of Bonner and Hoffman (1963) on *D. mucoroides.*

KOH at concentrations up to 20% had little or no effect on either dark- or light-incubated cultures. The reciprocal might have been expected based upon the above observation.

As in Bonner and Hoffman's experience, charcoal does increase the density of aggregations among myxamoebae incubated in the light. More interestingly, the presence of charcoal in dark-incubated cultures increased the number of centers to about four times the control value. This result would be consistent with the presence of a center-inhibiting factor, or suppressor, which exists, at least in part, in the gaseous phase.

Mineral oil provides the sharpest change in the behavior of the myxamoebae. In illuminated cultures, the density of aggregation was increased about three-fold (*cf.* Bonner and Hoffman, 1963), while in the dark there was a *14*-fold increase. That is to say, in the dark and under mineral oil a density of aggregation was reached which is normally achieved only in the light! How this effect is brought about is still unclear, but it could possibly result from the adsorption by mineral oil of a suppressive factor.

Growing myxamoebae in the light as opposed to darkness does not alter their behavior in terms of aggregative density.

Discussion

A number of hypotheses could be invoked to account for the effect of light on the initiation of cell aggregation and on aggregation density in *Polysphondylium pallidum.* Of these, only one was subjected to any sort of experimental evaluation. The major feature of this particular hypothesis is that light acts, in some way, to prevent a center-suppressing factor present in the environment from inhibiting aggregation. This might occur by light limiting the production of the inhibiting factor or perhaps by destroying it. Another possibility would be for light to render myxamoebae less sensitive to suppression or more sensitive to a lower level of stimulation. In any case, the net effect would be an increase in the number of centers among myxamoebae exposed to light. What, then, are the data supporting this hypothesis?

As evidence for the presence of a suppressor (in this case one existing in the gaseous phase), one may recall that an agent such as charcoal in both isolated and *in situ* populations increases, as does light, the density of aggregation centers. Furthermore, in cultures grown *in situ,* sorocarp formation and aggregation induced by charcoal follow much the same pattern as that of light induction, *i.e.,* in both a zone of morphogenetic activity is formed near the periphery of the expanding colony. Another piece of evidence, albeit indirect, is the appearance of sorocarps at or near the point of inoculation in cultures grown in the dark. Bonner and Hoffman (1963) discuss in some detail situations in which a relatively great air space above the myxamoebae permitted center formation where none might have occurred under more confined conditions, hence implying the accumulation of a center-limiting factor in the atmosphere. Cell aggregation which occurs at the point of inoculation before the culture has attained substantial dimensions is possibly a good example of this. Finally, cultures which are exposed to charcoal aggregate more quickly than do unexposed cultures—a response also seen when one compares light-treated cultures with those maintained in darkness.

A diminution in the effectiveness of light with time has been indicated by the experiments illustrated in Figures 1 and 2, and, indirectly, by the experiments presented in Figure 3. On the basis of the hypothesis presented above, this diminution could be accounted for by the accumulation of the suppressing factor(s). That is, the longer the culture remains in the dark, the more suppressor accumulates and the more difficult becomes the process of cell stimulation. On the other hand, if cultures are illuminated shortly after they cease feeding, *i.e.,* as soon as they become "ready" to aggregate, little suppressor would have accumulated, and, as a consequence, a small amount of stimulant, *e.g.* less light, could give rise to a comparatively great response. In fact, this is quite nearly the type of behavior represented in Figures 1 and 2 where the greatest sensitivity to light is evident early within the incubation period.

Is there evidence, beyond that already presented, for a suppressor type of hypothesis? Bonner and Hoffman (1963) found that the major species of the Dictyosteliaceae could be separated upon their aggregative behavior into two groups: gas-sensitive and gas-insensitive. In their experiments the gas-sensitive species, *Polysphondylium pallidum, P. violaceum,* and *Dictyostelium mucoroides,* showed vastly increased aggregation densities in the presence of charcoal and under mineral oil. *Dictyostelium purpureum* and *D. discoideum,* the gas-insensitive species, are invariant to these factors. Therefore, for at least three of the five major species there are indications of a center-limiting factor in the gaseous phase of the environment.

Snoopman (1963) found in *D. deminutivum* (*sp. nov.*, to be described) that cell aggregation and culmination were often retarded in standard Petri dish cultures, but proceeded normally when charcoal was added or when the glass lid was replaced by a porous clay top. Shaffer (1961) noted that light applied to dark-incubated cultures of *P. violaceum* brought about a burst of aggregative activity. This response normally occurred within 20 minutes after exposure, which correlates well with the quick response seen in *P. pallidum.* More germane are his observations on the aggregative behavior of myxamoebae sandwiched between two thin layers of agar. In the dark, cultures of this type usually either failed to aggregate or aggregation was delayed for periods of several days, *except* in those cultures which had trapped air bubbles, or in which the myxamoebae came to lie in close proximity to the edge of the overlying piece of agar. Here, again, the implication is for the presence of a center-suppressing factor which exists in the gas phase.

It would be well to point out another observation made by Shaffer (1961) and confirmed during the course of this investigation. Under agar, where centers are normally inhibited or their numbers reduced, myxamoebae are still capable of forming quite extensive streams. Thus, while center formation is impaired, the ability to undergo the physiological changes requisite to stream formation has not been impaired.

The factor postulated as being involved in center formation has been termed a "suppressor," which may be similar if not identical to Bonner and Hoffman's "spacing substance." The latter term was used to connote a gaseous substance which determines in some rather precise way the spacing of aggregation centers, *i.e.,* the distance between one center and another. During the present investigation, evidence has been obtained which points to the presence in *P. pallidum* of a gaseous center-suppressing factor that is produced by the myxamoebae and may determine whether a center *will* or *will not* develop. Nothing with certainty can be said as to whether or not this factor determines the distance between two centers. Because of this uncertainty, the rather specific term "spacing substance" has been avoided and the more general expression "suppressor" employed in presenting the results of this study.

As to the nature of the gas, Bonner and Hoffman (1963) have presented evidence that it may be CO_2. In this work, it has been found, in agreement with the above, that CO_2 will reduce the number of centers formed by a population of myxamoebae as, in contrast, charcoal will enhance the number. The only incongruity is that KOH, normally a good CO_2 absorbent, was only marginally effective in increasing the number of centers and then only in the dark (Table I). Consequently, at least for *P. pallidum* strain WS-320, it would seem unlikely that CO_2 is the naturally occurring center-suppressing factor.

Only a single explanation, the removal or inactivation of a suppressor, has been offered in this discussion to account for the effect of light on cell aggregation in *P. pallidum.* One need only to recall that two major species, *D. discoideum* and *D. purpureum,* are both light-sensitive *and* gas-insensitive to realize how inadequate is this simple explanation. We certainly cannot say at this time that the mechanism by which light effects aggregation in these two species may not be operative in *Polysphondylium pallidum* as well.

I wish to thank Professor Kenneth B. Raper for providing the laboratory facilities required for this investigation, and for his help and encouragement, particularly in the preparation of this manuscript.

This study was supported in part by a Training Grant from the National Institutes of Health (5-T1-GM-686).

Summary

Experiments have been performed on the effect of light on cell aggregation and aggregation density in *Polysphondylium pallidum*, strain WS-320. The results of these experiments may be summarized as follows:

1. Cell aggregation occurs more quickly and with a greater density of aggregation centers in cultures incubated in the light than in comparable cultures incubated in the dark.
2. Myxamoebae grown in the dark in liquid culture and deposited on agar are most sensitive to light within the first few hours of incubation.
3. Beyond the initial period of sensitivity, there is a diminution in response resulting in an almost complete absence of light-induced aggregation.
4. Little variation in response occurred when myxamoebae were exposed to light for periods of from one to 15 minutes, indicating a fairly sensitive light response mechanism.
5. In cultures grown *in situ*, exposure to the light following dark incubation induces aggregation in two- to three-day-old myxamoebae. Older myxamoebae fail to display any sign of morphogenetic activity in response to light; they do respond, however, in the presence of charcoal.
6. Of certain physical and chemical agents tested for their effect on aggregation, charcoal and mineral oil were found to increase aggregation density in both light and dark, while CO_2, in the light, had the opposite effect.

The similarity in response achieved by charcoal and mineral oil, and by light, suggests that the action of light may be to change the relationship between aggregation and a center-suppressing factor that accumulates in the environment. In the light, aggregation occurs possibly because light limits the production of or destroys the center-suppressing factor, or renders the cells less sensitive to it. In the absence of light, aggregation may be inhibited by this substance while the cells are shunted in the direction of microcyst formation. If, in the latter situation, charcoal or mineral oil were added prior to actual cyst formation, the suppressor would be absorbed and aggregation could occur.

LITERATURE CITED

Blaskovics, J. C., and K. B. Raper, 1957. Encystment states of *Dictyostelium*. *Biol. Bull.*, **113**: 58–88.

Bonner, J. T., 1947. Evidence for formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.*, **106**: 1–26.

Bonner, J. R., and M. R. Dodd, 1962. Aggregation territories in the cellular slime molds. *Biol. Bull.*, **122**: 13–24.

Bonner, J. T., and M. E. Hoffman, 1963. Evidence for a substance responsible for the spacing pattern of aggregation and fruiting in the cellular slime molds. *J. Embryol. Exp. Morph.*, **2**: 571–579.

Gerisch, Günther, 1960. Zellfunktion und Zellfunktionswechsel in der Entwicklung von *Dictyostelium discoideum* I Zellagglutination und Induktion der Fruchtkörperpolarität. *Archiv f. Entw.*, **152**: 632–654.

Harper, R. A., 1932. Organization and light relation in *Polysphondylium*. *Bull. Torrey Bot. Club*, **59**: 49–84.

Hohl, Hans-Rudolf, and K. B. Raper, 1963. Nutrition of cellular slime molds. I. Growth on living and dead bacteria. *J. Bacteriol.*, **85**: 191–198.

Olive, L. S., and C. Stoianovitch, 1960. Two new members of the Acrasiales. *Bull. Torrey Bot. Club*, **87**: 1–20.

Potts, G., 1902. Zur Physiologie des *Dictyostelium mucoroides*. *Flora*, **91**: 281–347.

Raper, K. B., 1940. Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.*, **56**: 241–282.

Raper, K. B., 1951. Isolation, cultivation, and conservation of simple slime molds. *Quart. Rev. Biol.*, **26**: 169–190.

Shaffer, B. M., 1958. Integration in aggregating cellular slime moulds. *Quart. J. Mic. Sci.*, **99**: 103–121.

Shaffer, B. M., 1961. The cells founding aggregation centres in the slime mould *Polysphondylium violaceum*. *J. Exp. Biol.*, **38**: 833–849.

Shoopman, J., 1963. *Dictyostelium deminutivum*, sp. nov. A new cellular slime mold from Mexican soils. M. S. Thesis, U. Wisconsin.

THE INTERMOLT CYCLE OF AN ANOMURAN, PETROLISTHES CINCTIPES RANDALL (CRUSTACEA–DECAPODA)

N. G. KURUP[1]

Department of Biology, University of Oregon, Eugene, Oregon

The concept of the intermolt cycle in crustaceans as a well-defined sequence of stages was first advanced by Drach (1939) on the basis of his classical studies on the Roscoff population of brachyurans. His subsequent work (Drach, 1944) on the macruran, *Leander serratus,* confirmed that there is a certain amount of generality for this concept in decapod Crustacea. He describes three major phases of transformation between successive molts: (1) the postmolt—a period of marked turgescence by absorption of water (2) the intermolt—a phase of calcification of the skeleton and tissue growth, and (3) the premolt—a period of active preparation for the next molt. Each of these phases is further subdivided into stages of shorter duration based on specific morphologic features. Hiatt (1948) wrote a monograph on the biology of *Pachygrapsus crassipes* including the diagnostic changes in the molt cycle, and Kincaid and Scheer (1952) proposed a key for staging another Pacific shore crab, *Hemigrapsus nudus.* Scheer (1960) followed up the work and presented criteria for the molt stages of Macrura by studies on three species of caridean Natantia of the Mediterranean coast. Next to nothing is known of the intermolt cycle of Anomura and the present study extends the criteria to this group of Malacostraca. In a recent paper (Kurup, 1964) the author has indicated, that, compared to macrurans and brachyurans, the details in the genesis of the carapace are somewhat different in the porcelain crab, *Petrolisthes cinctipes,* and that the uropods are the best guide in staging it.

MATERIAL AND METHODS

The crabs were collected at low tide from Cape Arago, Oregon, and maintained in a constant-temperature aquarium. As environmental changes are known to affect molting (Passano, 1960; Kurup, 1963), care has been taken to approximate normal living conditions for the crabs. Rocks and sea weeds were planted in tanks to simulate natural habitat. The temperature was kept constant at 13° C. The crabs were subjected to an average 14-hour diffuse illumination per day from fluorescent lamps that lighted the whole aquarium room with luminosity of 18 to 22 foot candles in the tanks. The lamps were controlled by automatic timers.

Petrolisthes is an algal feeder and a scavenger. The animals were fed on the green alga, *Cladophora,* or on the organic detritus of the sea weed, *Fucus,* which, when in a state of suspension in water, is triturated by the maxillipedal bristles and ingested by the crabs. The salinity of the sea water in the tanks was kept at 35 ± 3‰.

---

[1] Professor of Zoology, Mahatma Gandhi College, Trivandrum, Kerala, India.

Morphological features to characterize stages in the intermolt cycle were studied in uropods. Permanent mounts of the uropods were made after fixation in Bouin's fluid or Carnoy's mixture and staining either by Ehrlich's hematoxylin or by magenta (basic fuchsin). Temporary preparations for immediate examination were made in *Cancer* saline (Robertson, 1939) or plain sea water.

The classification of the intermolt cycle of *Petrolisthes* is based on the Drach stages for Brachyura and the notations designating the stages introduced by Drach (1939) are followed. Accordingly, the postmolt period is subdivided into four stages, A_1, A_2, B_1 and B_2, the intermolt period into C_1, C_2, C_3 and C_4, and the premolt period into D_1, D_2, D_3 and D_4.

Though the behavioral and morphological changes in the stages of the intermolt cycle of *Petrolisthes* broadly conform to those of Macrura and Brachyura, the duration of the various stages and the characters used in their recognition vary widely. The behavior pattern reported, of animals in the different stages, is primarily based on studies in the field.

The duration of the stages of the intermolt cycle was calculated on a mean value basis of observations on captive animals. Two groups of crabs of 12 each (6 males and 6 nonovigerous females), all in B_2 at the beginning of the observations, were selected for this purpose from collections on June 27 and September 3, 1963. The crabs chosen were all of the same size, measuring 1.2 cm. carapace width, and individual variations in the time between molts were negligible. Estimation of the differential sclerotization of the sternites and appendages other than the chelipeds has not been attempted; such an assessment would be somewhat arbitrary as the sternites are not extensive and the walking legs are much too small to explore by palpation. The chelae, both ambulatory and prehensile, are very large in *Petrolisthes* and the changes in them and the dorsal half of the carapace alone are considered in terms of chitinization of the integument.

Observations and Discussion

Morphological and behavioral signs and the span of the intermolt cycle

As in the case of other decapods, during the early postmolt (A_1 and A_2 stages) the exoskeleton of the porcelain crab is completely soft, and the carapace has a bright reddish brown color. Due to water absorption, the size and weight of the body in A_1 are fluctuating. In A_1 the animal is extremely quiescent also; it does not feed and cannot lift its body on its legs. Such "soft crabs" collected from the coast usually nestle under rocks in tide pools and are never found exposed at any time. In A_2 the crab moves with difficulty, but is still inactive and nonfeeding. Stage A_1 lasts for about 24 hours and A_2 for 48 to 72 hours.

The late postmolt stages last for about 5 days, the time spent in B_1 is 60 to 72 hours and that in B_2 about 48 hours. Smaller crabs start feeding only in B_2 while larger animals move in search of new feeding grounds even in B_1. In B_1 the protogastric area has become hard, the other parts of the carapace being firm but easily depressible. In the B_2 stage the mesogastric area also attains rigidity. The chelipeds, of which the carpus and propodus were supple in B_1, assume uniform hardness in B_2.

The intermolt stages are relatively of longer duration in *Petrolisthes;* in *Pachygrapsus,* they run from 32 to 50 days (Hiatt, 1948), in *Cancer* for many months (Drach, 1939) and in natantians from 29 to 45 days. Drach (1944) suggested that the proportionate number of animals at a certain stage in one collection can be considered an approximate index of the duration of that stage, and Scheer (1960) has found it a reliable clue for calculating the time run for each stage of the intermolt cycle in natantians. But, in *Petrolisthes* this principle was not found accurately applicable. For, it may be that, unlike natantians which are netted, equal chance and facility for the collection of the different stages of *Petrolisthes* are quite remote, since the porcellanids are under-rock forms, some invariably escaping the collector during hand-picking.

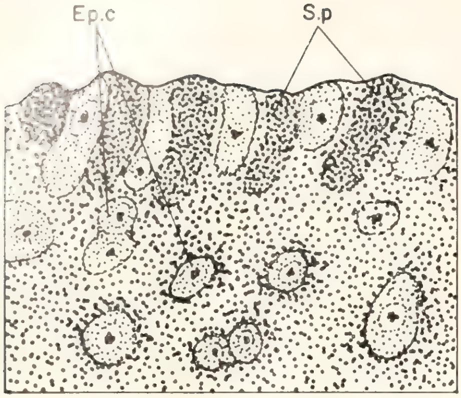
As indicated above, the intermolt period is quite extensive in *Petrolisthes* in spite of the small body size of the crab, the mean time recorded in laboratory animals being 107 days with a standard error of 21. However, there were three animals in the samples which did not molt at all and which died on the 122nd day, possibly of accidental salinity stress in the tanks. Two were still in C_4 and one in D_2 when they died. As age is an important factor in molting, it is possible that in the former molting had ceased altogether and that in the latter senility would have retarded the process. The break-up in the span of the C stages is as follows: C_1—13 days; C_2—24 days; C_3—26 days; C_4—44 days (or more).

At the beginning of the intermolt, the crabs have attained the characteristic dark tan color for the carapace. In C_1 the urogastric area of the carapace has also become rigid, the anterior and posterior branchial areas and the cardiac zone being firm but still depressible. In C_2 the branchial areas have become uniformly rigid, but the cardiac area is still slightly depressible. It is only in C_3 that the dorsal carapace becomes completely hard and this hard texture is retained through D_1.

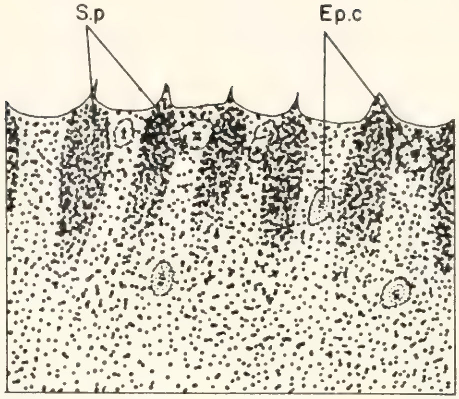
The early premolt is an active phase for the crabs; in D_3 a general debility sets in, and the animals seem to cease feeding and locomotion. Both D_1 and D_2 last for 4 to 5 days each; and D_3 for less than 72 hours. D_4 is comparatively short and extends for 12 to 24 hours only. In the late D_1 the carapace begins to soften and the urocardiac area can be slightly depressed. In D_2 the entire cardiac area of the carapace has become thin and depressible, evidently by the resorption of material from the old cuticle. This thinning of the cardiac area is continued through D_4 when the carapace becomes so fragile as to crack by touch through the ecdysial line. Exuviation appears really strenuous for the crab. Even after the rupture of the dorsal carapace through the epimeral suture, the crab devoid of a support takes about 6 to 8 hours to wriggle out of its exuvium, and the time consumed is predominantly for withdrawal of the chelipeds from the exoskeleton. If the animal has been provided with a log or stone as substrate, the process is quickened and exuviation is over in about two hours time.

Stratification of new epidermis

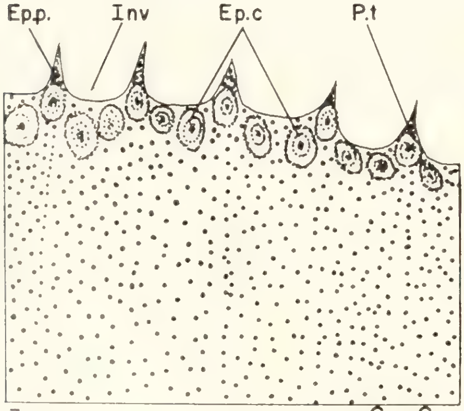
As in the natantian, *Processa edulis,* the beginnings of integumentation are noticeable in the porcelain crab in the appendages even in stage C_1, the early intermolt (Fig. 13). It may be noted, however, that in Brachyura the process is delayed until C_4 or D_1 (Drach, 1939; Hiatt, 1948; Kincaid and Scheer, 1952). In *Petrolisthes,* the epidermis that is newly formed in C_1 shows sporadically dis-



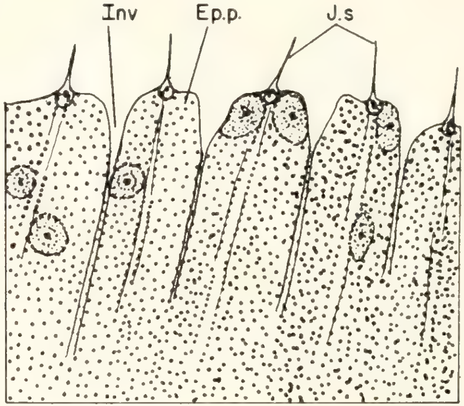
1 C_1



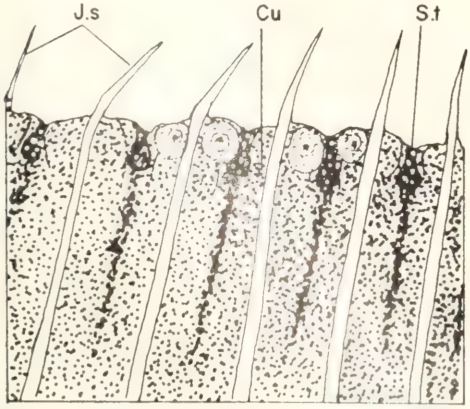
2 C_2


Ep.p.
Inv
Ep.c
P.t

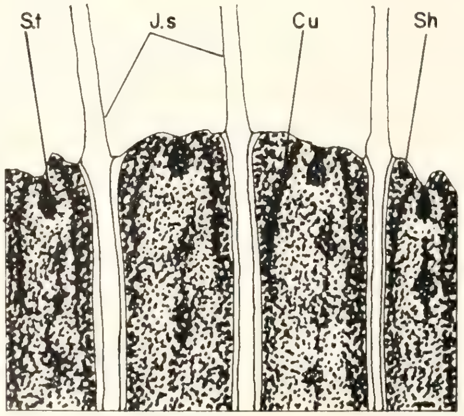
3 C_3-C_4



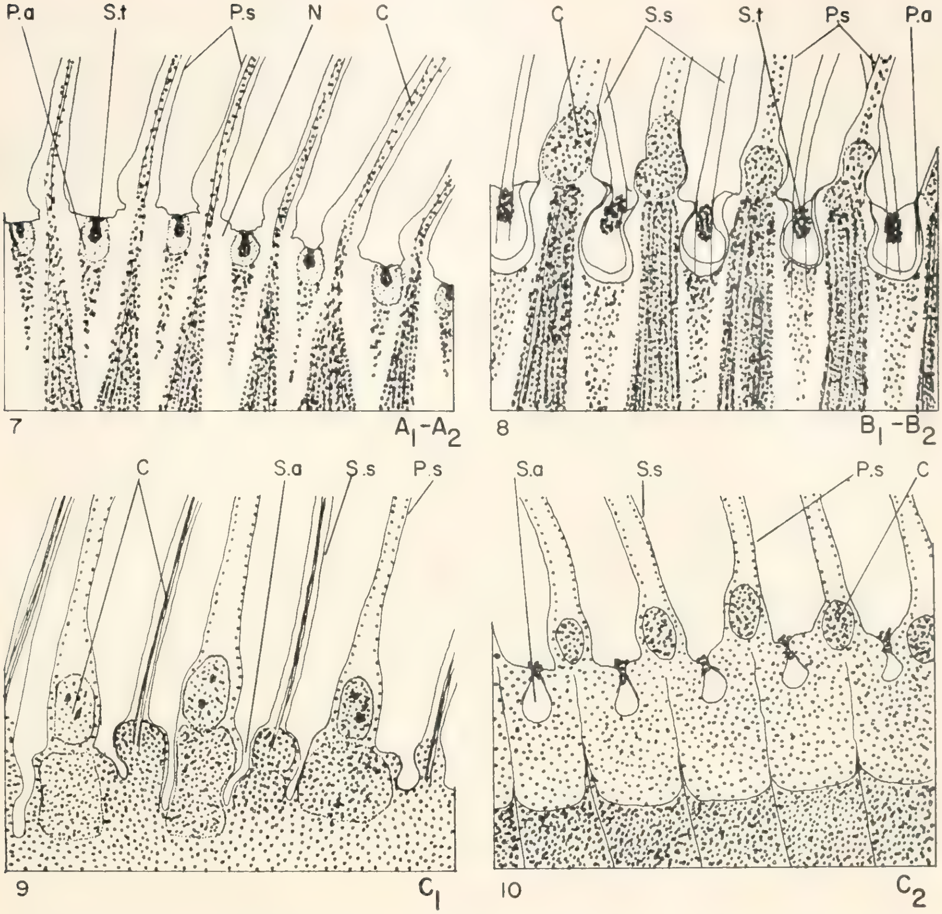
4 D_1-D_2



5 D_3

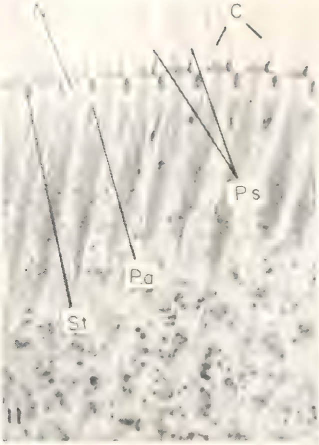


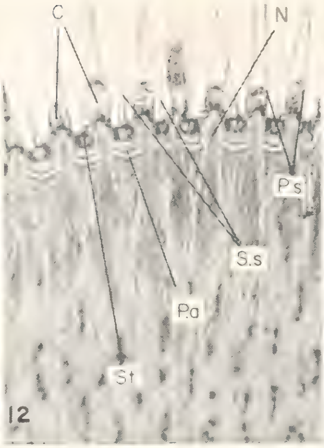
6 D_4

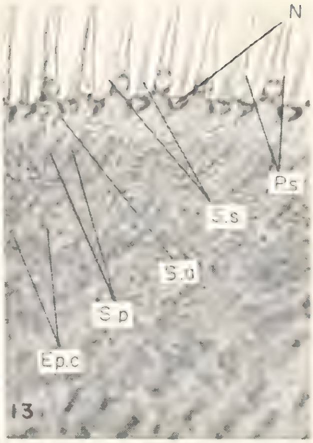


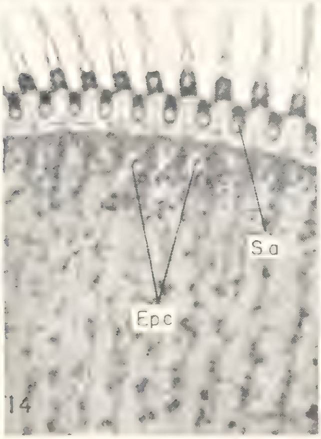
FIGURES 1 to 10. Diagrammatic representation of epidermal stratification and setogenesis in the uropods of *Petrolisthes cinctipes*. The respective stages are indicated on the drawings. C, Cone; Cu, Cuticle; Ep, Epidermis; Ep.c, Epidermal cell; Ep.p, Epidermal papilla; Ex.s, Exuvial setae; Inv, Invagination; J.s, Juvenile setae; N, Node; P.a, Primary alveolus; P.s, Primary setae; P.t, Primary trichogen; S.a, Secondary alveolus; S.p, Setal primordium; S.s, Secondary setae; S.t, Secondary trichogen; Sh, Setal sheath.

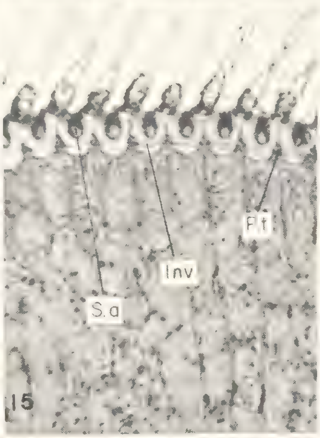
tributed epithelial cells, some in various stages of mitosis (Figs. 1 and 13). Correlated with the development of the bristles, the epidermis undergoes some significant morphological changes. The epidermal layer closely adheres to the exoskeleton until C_3. It becomes somewhat amber-colored and separable from the cuticle (not without damage) only in C_4. It seems to be lined by a membrane apparently formed by the condensation of some kind of secretion discharged by the epidermal cells. In the uropods of C_3, this limiting membrane furnishes a well defined boundary for the new epidermal stratum from the old (Fig. 14). This layer might correspond to the "couche membraneuse" characteristic of the C_4 stage of *Cancer pagurus* which Drach (1939) described.

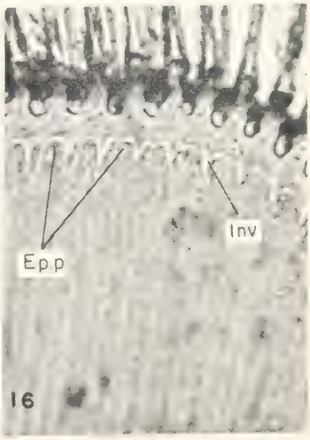

A
C
Ps
P.a
St
11

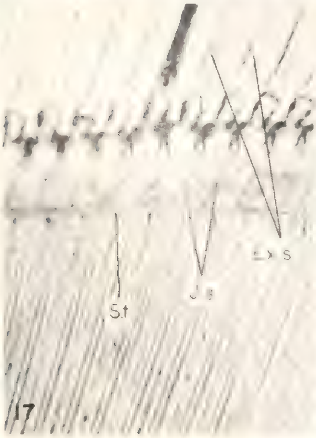

C
N
Ps
S.s
P.a
St
12

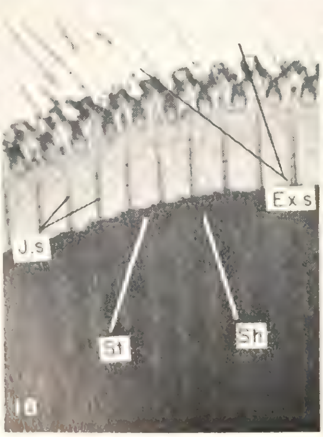

N
Ps
S.s
S.a
S.p
Ep.c
13

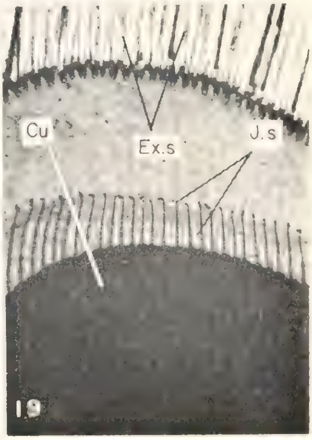









Ex.s
J.s
St
Sh
18



FIGURES 11 to 18. Photomicrographs of uropods in A_2, B_2, C_1, C_2, C_4, D_1, D_3 and D_4 stages, respectively, showing the development of the epidermis and setae during the intermolt cycle of the porcelain crab. Magnification × 150. Abbreviations as before.

FIGURE 19. Photomicrograph of the uropod of a D_1 crab, showing an individual variation, wherein the secondary setae have already appeared. Magnification × 70. Letterings as before.

In C_4 invaginations appear in the epidermis—an important initial step in bristle development (Figs. 3 and 15). The formation of these foldings might be due to the expansion of the membranous layer, and as Wigglesworth (1933) pointed out for the blood-sucking bug *Rhodnius*, the membrane expands perhaps by the imbibition of water, which might be provided from the body fluid of the crab. The invaginations become deep and narrow in D_1 (Fig. 16) and the layer develops longitudinal pleats also with epidermal papillae through which the setae sprout (Figs. 4 and 16). This forms a clear-cut distinction between C_4 and D_1 stages. By D_2 chitinization of the integument has started. The new epidermis of the uropods prior to stage D_2 dissolves away on boiling in aqueous saturated KOH, which suggests that chitin deposition begins only in D_2. As stated years ago by Vitzou (1882), in the cuticular development of Crustacea, prior to chitin deposition, separation of this polysaccharide is effected within the bodies of the cells themselves. In the D_2 stage of *Petrolisthes* the epidermal furrows become deeper and by D_4 the new cuticle is considerably strengthened by the progressive impregnation of chitin, the old cuticle shrinking in the meantime, ready to be cast off (Figs. 18 and 19).

Setogenesis—a concept

The setae are hollow cuticular outgrowths, distributed mainly on the appendages of crustaceans. Generally the development of new bristles is started during the early premolt of the intermolt cycle prior to the secretion of chitin in the integument (Drach, 1939; Scheer, 1960). The formation of setae in the different appendages is also not simultaneous in Brachyura and Macrura; in certain parts it is precocious and in others it is delayed. Such a chronological variation in bristle formation and growth is true of *Petrolisthes* too. Nevertheless, in this anomuran, an orderly sequence in the development of setae between molts—which may be called setogenesis—is in evidence, and serves as a reliable criterion for the definition of the stages of the intermolt cycle. In the uropods of the porcelain crab, the bristles that fringe the tip are long and those on the sides are small and spinous. Setogenesis was followed up in relation to the various stages of the intermolt cycle by microscopic examination of the longer bristles of the uropods, which is possible even without removing the appendage from the abdomen of the live animal.

The initiation of new setae is relatively early in the anomuran, *Petrolisthes*, compared to brachyurans and macrurans. Concomitant with the definition of the new epidermis, setal primordia appear as localized patches in C_1, and in C_2 and in C_3 they become increasingly emphasized (Figs. 1, 2, 13 and 14). The composition of the primordium is in evidence in C_4; each appears to be made up of a few epidermal cells, of which one is larger and hence more prominent. This large cell is the hair-forming cell or trichogen (after Wigglesworth, 1933), and as more than one set of formative cells appear, the first formed cells may be called primary trichogens (Figs. 3 and 15). From the trichogens fibrillar prolongations that form the basis of the setae emerge, visible even in C_3 (Fig. 14). In their exhaustive study on the development of the bristles in *Drosophila*, Lees and Picken (1945, p. 413) stated that "the trichogen produces the cytoplasm throughout bristle development, so that the whole bristle is in a sense an exploded epidermal cell."

In C_4 invagination takes place from the edge of the new epidermis, and the trichogens, with their juvenile setae, that appear as conical elevations or papillae, flank the sides of the epidermal sockets (Figs. 3 and 15). The invagination goes deeper in D_1 and D_2 (Fig. 16). The setae appear to grow both at their tips and at their bases and they develop a long shaft and become well set in their corresponding furrows in D_2. By D_3 the setae become clearly defined as tubular spines buried in their respective crypts, their tips all emerging out of the new epidermal stratum (Figs. 5 and 17). They are also directed toward the exuvial setae, the older bristles that stay on till exuviation. The new set of setae thus formed, which may be called primary setae, become densely chitinized and disposed in line with the exuvial setae in D_4 (Fig. 18). They also rest in setal sheaths that appear to have been derived from the epidermal invaginations (Figs. 6 and 18). In *Petrolisthes* the primary setae do not extend into the core of the exuvial setae, although Scheer (1960) has reported the extension of new setae within the cuticle of the old in *Leander* and *Processa*. In the final phase of premolt (D_4), the primary setae do not show any morphological change, but become more closely set and appear steadily drawn away from the exuvial setae (Fig. 19) as a result of the muscular movements that facilitate exuviation.

The primary setae of the "soft crab" (stages A_1 and A_2) are different from those of the D_4 stage in that they have developed internal cones extending longitudinally in relation to the setal axis. The base of the bristle is demarcated by a "node," and in A_2 the nodes and cones are more clearly delimited (Figs. 7 and 11). Besides, in A_1, epidermal notches or alveoli appear between the primary setae, each alveolus enclosing a trichogen—the secondary trichogen—for the generation of a second set of bristles (Figs. 7 and 11). The *anlagen* of the secondary trichogens appear as early as D_3 (Figs. 5 and 17).

The alveoli of the postmolt (A_2), which may be called primary alveoli, become somewhat constricted in B_1; in B_2 secondary setae sprout from their trichogens, grow long and emerge as tubular spines between the primaries (Figs. 8 and 12). Sometimes more than one seta may arise from one secondary trichogen. As Wigglesworth (1933) holds for *Rhodnius*, the seta-forming process appears to give a thrust and to penetrate through the trichogen.

A precocious development of the secondary setae was observed in one experimental animal (nonovigerous female) wherein the secondaries had already arisen in the D_1 stage, in the uropods (Fig. 19). However, the timing and other details of setogenesis in this crab appeared just the same as in other animals.

In B_2 the plasmatic cones of the setae are shrunk and have become more or less withdrawn within the setal cavity (Figs. 8 and 12). This configuration of the cones is retained all through the subsequent stages. The difference in the size and shape of the primaries and the secondaries is conspicuous and characterizes stages B_2 and C_1. By C_1 the secondaries also acquire well defined nodes at their bases (Figs. 9 and 13), but still they are thin and fibrillar when compared to the primaries. In late C_1 the cones also appear within the secondaries, but they remain as an axial reinforcement only for a short time, and by C_2 they shrink and become withdrawn toward the nodes. Again in C_1, the formation of secondary alveoli is evident and in C_3 they are better defined (Figs. 9, 10, 13 and 14). However, trichogens are not generally noticeable within them. The morphological

TABLE I

Key for staging Petrolisthes cinctipes

Stage	Duration	Morphological (including morphogenetic) features			
		Carapace	Cheliped	Epidermis	Setae of uropods
A_1	About 24 hours	Completely soft	Very soft	Cuticularized	Primary alveoli and secondary trichogens appear between setae; cones and nodes forming.
A_2	48 to 72 hours	Soft	Soft	As above	As above, but the cones and nodes are well-defined
B_1	60 to 72 hours	Protogastric area only hard	Carpus and propodus supple	As above	As before, but the alveoli are narrow
B_2	About 48 hours	Proto- and mesogastric areas hard	Uniformly hard	As above	Secondary setae sprout; cones shrink to base; two sets of setae clear
C_1	13 days	Proto- and mesogastric areas and anterior branchial zone hard	As above	New epidermis formed as a membranous layer	Secondaries also develop nodes; Setal primordia appear in new epidermis
C_2	24 days	Hard except cardiac area which is firm but depressable	As above	Membranous layer better defined	Primary and secondary setae indistinguishable
C_3	26 days	Uniformly hard	As above	Stratification very clear	Secondary alveoli develop between setae
C_4	44 days (or more)	Very hard	As above	Invaginations appear with conical papillae on sides	New setae just sprouting from epidermal papillae
D_1	4 to 5 days	Urocardiac area depressable	As above	Epidermal papillae clearly demarcated	New setae grow out from papillae
D_2	4 to 5 days	Entire cardiac area depressable	As above	Invagination deepened; sclerotization started	New setae fibrillar and become well set in furrows
D_3	About 3 days	Generally thin; cardiac area cracks if pressed too hard	Not as hard	Chitinization continued; new cuticle easily separable	Setae spinous; *anlager* of secondary trichogens appear
D_4	About one day	Thin and breaks easily	As above	Integument dark brown and remains detached from old	As above, but setae lined by sheaths in epidermis

distinction between the primary and secondary setae vanishes in C_3 and the primaries of the next cycle have already emerged as rudiments from the newly formed epidermis (Figs. 10 and 14).

Generally, the secondary alveoli do not appear to contain trichogens. This evidently means that a tertiary set of setae does not emerge in spite of the alveoli, as the metabolic processes are directed toward the development of the primaries of the succeeding setal cycle. Nonetheless, there are instances wherein the tertiaries have also emerged and it is likely that such a situation would be typical of animals that enter anecdysis, where a new epidermis does not stratify as the molting has stopped altogether.

This work has been supported by grants G-21448 from the National Science Foundation of the United States and PL-584 from the Fulbright Commission of India, and was carried out under the supervision of Professor Bradley T. Scheer. The author records his grateful appreciation to Dr. Scheer for his help and criticism throughout the work. The author is also thankful to Miss Gillian Shane (Scripps Institution of Oceanography, La Jolla, California) for her assistance in collection and study of the animals in the field.

Summary and Conclusions

1. The intermolt cycle of *Petrolisthes cinctipes* is described and morphologic criteria are proposed for designating the physiologic stages of the cycle. The key for staging the animal is provided in Table I.

2. The chronological changes in the derivation of new epidermis and bristles in the uropods furnish a reliable identification of the various stages even without recourse to palpation of the exoskeleton. The characteristics described by Drach (1939, 1944) and Scheer (1960) for certain species of crabs and prawns are found useful in staging a wide variety of Brachyura and Macrura; likewise, the diagnostic features in the stages of the porcellanid might be helpful in staging other Anomura as well.

3. In the porcelain crab the duration between two successive molts is unusually long (about 115 to 130 days), especially in view of the small body size of the animal. This might mean that the growth rate of this crab is relatively higher. It would be equally interesting to know of a possible seasonal shortening or lengthening of the intermolt cycle (see Scheer, 1960), the molting periodicity and the total number of molts the crab passes through before it enters the terminal anecdysis.

LITERATURE CITED

Drach, P., 1939. Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Oceanogr. Inst. Paris N. S.*, **19**: 103–391.

Drach, P., 1944. Étude préliminaire sur le cycle d'intermue et son conditionnement hormonal chez *Leander serratus* (Pennant). *Bull. Biol. France et Belg.*, **78**: 40–62.

Hiatt, R. W., 1948. The biology of the lined shore crab, *Pachygrapsus crassipes* Randall. *Pacific Sci.*, **2**: 135–213.

Kincaid, F. T., and B. T. Scheer, 1952. Hormonal control of metabolism in crustaceans. IV. Relation of tissue composition of *Hemigrapsus nudus* to intermolt cycle and sinus gland. *Physiol. Zool.*, **25**: 372–380.

Kurup, N. G., 1963. Crustacean hormones. *J. Anim. Morph. Physiol.,* **10**(2) : 113–149.

Kurup, N. G., 1964. The incretory organs of the eyestalk and brain of the porcelain crab, *Petrolisthes cinctipes* (Reptantia-Anomura). *Gen. Comp. Endocrinol.,* **4**(1) : 99–112.

Lees, A. D., and L. E. R. Picken, 1945. Shape in relation to the fine structure in the bristles of *Drosophila melanogaster. Proc. Roy. Soc. London. Ser. B,* **132**: 396–423.

Passano, L. M., 1960. Molting and its control. *In:* The Physiology of Crustacea. Vol. I, T. H. Waterman ed. Academic Press, New York, pp. 473–536.

Robertson, J. D., 1939. The inorganic composition of the body fluids of three marine invertebrates. *J. Exp. Biol.,* **16**: 387–397.

Scheer, B. T., 1960. Aspects of the intermolt cycle in natantians. *Comp. Biochem. Physiol.,* **1**: 3–18.

Vitzou, A. N., 1882. Recherches sur la structure et la formation des téguments chez les Crustacés Décapodes. *Arch. Zool. Exp. et Gen.,* **10**: 451–576.

Wigglesworth, V. B., 1933. The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera), with special reference to the function of the oenocytes and of the dermal glands. *Quart. J. Micr. Sci.,* **76**: 269–318.

THE SALINITY TOLERANCES OF SOME ESTUARINE PLANKTONIC CRUSTACEANS[1]

JOAN LANCE

Department of Zoology, University of Hull, England

In estuaries, copepods are important members of the zooplankton and benthos, yet there is little experimental information on the extent to which these crustaceans can withstand dilution. Data are available for a few free-living copepods (Marshall *et al.,* 1935; Zinn, 1942; Ranade, 1957; Eltringham and Barnett, 1958; Barnett, 1959; Hopper, 1960; Matutani, 1962; Battaglia and Bryan, 1964) and recent work on female *Acartia* adults shows that closely related planktonic species can vary considerably in their ability to survive in waters of low salinity (Lance, 1963). Thus, it has been established that *A. tonsa* has a greater tolerance to dilution than *A. bifilosa* which is in turn more tolerant than *A. discaudata.* These results are particularly relevant to the field work of Jeffries (1962) who deduces that both temperature and salinity can be important factors influencing the seasonal succession of *Acartia* species. It is possible that certain young or spawning stages are more sensitive and have narrower survival limits than non-spawning adults (Bullock, 1958). An attempt has therefore been made to compare the salinity tolerances of male and female adult copepods and of young stages in the life-history of copepod, decapod and cirripede species. Animals were taken from Southampton Water, which receives a flow of fresh water from three rivers, and investigated in the laboratory.

MATERIALS AND METHODS

Tow-nettings were taken from Southampton Water during the high tides, transported to the laboratory, and stored in Plymouth sea water (salinity 36.0–36.9‰) at a temperature close to that recorded in the estuary at the time of collection. The following animals were sorted from the nettings: adults and occasionally copepodites of various copepod species, namely *Acartia bifilosa* (Giesbrecht), *A. clausi* Giesbrecht, *A. discaudata* (Giesbrecht), *A. tonsa* Dana, *Centropages hamatus* (Lilljeborg), and *Temora longicornis* (Muller); young developmental stages of the decapod crabs *Carcinus maenas* Pennant and *Porcellana longicornis* (Linnaeus) and of the cirripede *Elminius modestus* Darwin. Sorted animals were fed, maintained at field temperature, and finally used for experiments 24 hours after capture.

All adult copepods (including the potential carnivores) were fed with the green flagellate, *Dunaliella,* and no animal food was provided. Larvae of the copepods and of *Elminius* were given the diatom, *Phaeodactylum,* as food. All plant cells were cultured in Erdschreiber medium (Gross, 1937) and only cultures in the exponential phase of growth were used. *Porcellana* zoeae were provided with pieces of *Mytilus* mantle together with *Phaeodactylum,* whereas the post larvae were given *Mytilus* mantle only. Although plant cells and live animal food are suitable for *Carcinus* zoeae, only *Phaeodactylum* was available. *Mytilus* tissue was fed to the megalopa stages of *Carcinus.*

---

[1] These studies were supported by a Research Studentship awarded by the Department of Scientific and Industrial Research. The author is most grateful to Professor J. E. G. Raymont of Southampton University for assistance and departmental facilities.

The salinity tolerance (*i.e.,* the ability to withstand dilution) of a species was determined according to the method described by Lance (1960, 1963). In each experiment, groups of animals were taken from full-strength sea water and placed directly into various dilutions. Each dilution, which can be expressed as a percentage of full-strength sea water, differed from its immediately higher or lower salinity in the range by 10%, 5%, or occasionally 2.5% sea water. Survival of the animals in diluted sea water was compared with that of individuals which were retained in full-strength sea water throughout. For each salinity, usually 50 animals were placed in 200 ml. of water. In the experiments using copepodites, however, sorting was very difficult and the numbers of larvae put in each vessel varied. Dead specimens were counted and removed at intervals, the first inspections being made after 20 hours. Preliminary tests had shown that there was little variation in survival between multiple runs and therefore only a single determination was made in each experiment. Plant food, when used, was maintained at a concentration of about 100,000 cells/ml. *Acartia* is most tolerant of dilution when the experimental and prevailing field temperatures are close, and least tolerant when the two temperatures differ markedly (Lance, 1963). Laboratory temperatures were therefore kept close to those recorded in the estuary at the time of collecting tow-nettings.

RESULTS

The range of dilutions causing deaths was identified for each species by observing the general trends in survival for the entire experimental period. Experiments lasted for three to 14 days, and in the longest runs, the general results obtained after three days rarely differed, and then only slightly, from those recorded later. Hence, although the period of observation varied, the results of different experiments can be readily compared. In each experiment, survival in 100% sea water and in diluted sea water at the upper end of the salinity range was similar. At the lower salinities, however, deaths associated with dilution effects occurred. The number of deaths progressively increased according to the degree of dilution and no individuals survived at the lowest salinities. The survival of animals in undiluted sea water was generally good. The full details of results obtained for each species are reported elsewhere (Lance, 1960).

Those dilutions which caused the death of at least 50% of the animals during the initial 20 hours of exposure are arbitrarily termed "lethal" salinities. The following formula has been used for calculating survival values for this 20 hour period:

$$\%\text{ survival in diluted sea water} = \frac{a_1}{b_1} \times \frac{b_2}{a_2} \times 100$$

where a_1 = number of survivors in diluted sea water, a_2 = number of animals initially placed in diluted sea water, b_1 = number of survivors in full-strength sea water, b_2 = number of animals initially placed in full-strength sea water.

Adult copepods of both sexes

Results are given for adult males and females of *Acartia discaudata, A. clausi,* and *Centropages hamatus* in Table I. For each species, a wider range of salinities was lethal to the males, indicating that they were less tolerant of dilution than the females. Furthermore, the range of salinities causing mortality was greater for the males of both *Acartia* species, though *Centropages* showed no difference between sexes. The consistently higher survival of the females is emphasized in Figure 1, which compares the data obtained for copepods after they had been exposed to dilutions at the lower end of the salinity range for 20 hours.

TABLE I

Salinity tolerances of copepods

Species	Stage in life-history	Experimental temperature (° C.)	Range of salinities causing mortality (% sea water)	Range of lethal salinities (% sea water)
Acartia discaudata	adults			
	female	16.0	0–35	0–25
	male	16.0	0–40	0–30
Acartia clausi	adults			
	female	15.2	0–45	0–35
	male	15.2	0–55	0–45
Centropages hamatus	adults			
	female	13.5	0–55	0–35
	male	13.5	0–55	0–45
Acartia tonsa	adults			
	female	17.0	0–25	0–10
	copepodites Stage I, II, III, IV	17.0	0–25	0–20
Acartia bifilosa	adults			
	female	10.0	0–40	0–20
	copepodites Stage I + II	10.0	0–40	0–20

The results for *Centropages* are unusual in that this is the only copepod species which survived less well in full-strength sea water than in dilutions at the upper end of the salinity range. Thus, very few deaths (90–96% survival after 8½ days) occurred in a salinity range of 90–60% sea water whereas only 60–62% of the copepods survived in undiluted sea water.

Copepodites and adult female copepods

The salinity tolerances of *Acartia tonsa* and *A. bifilosa* copepodites are compared with those of adult females in Table I. For *A. tonsa,* a smaller range of dilutions was lethal to adults than to Stage I, II, III, or IV copepodites, but otherwise each species yielded similar results for its adults and larvae. Differences become appar-

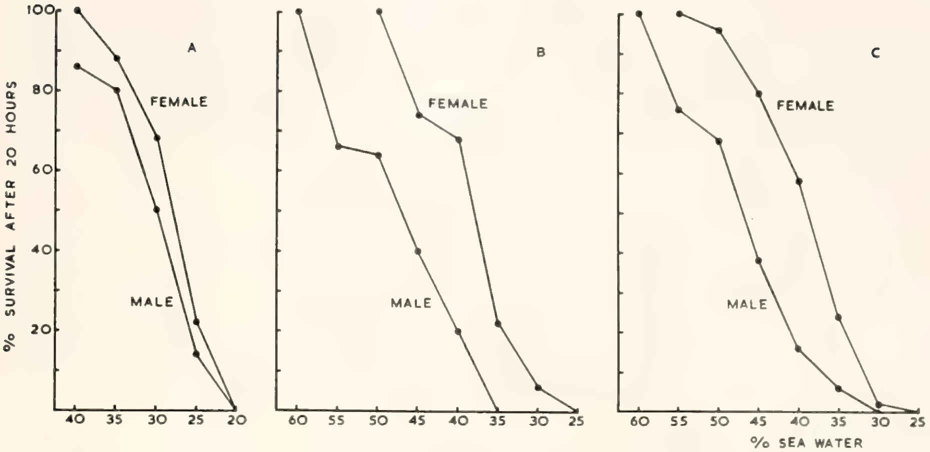


FIGURE 1. Per cent survival of adult copepods after 20 hours in diluted sea water. A, *Acartia discaudata*. B, *A. clausi*. C, *Centropages hamatus*.

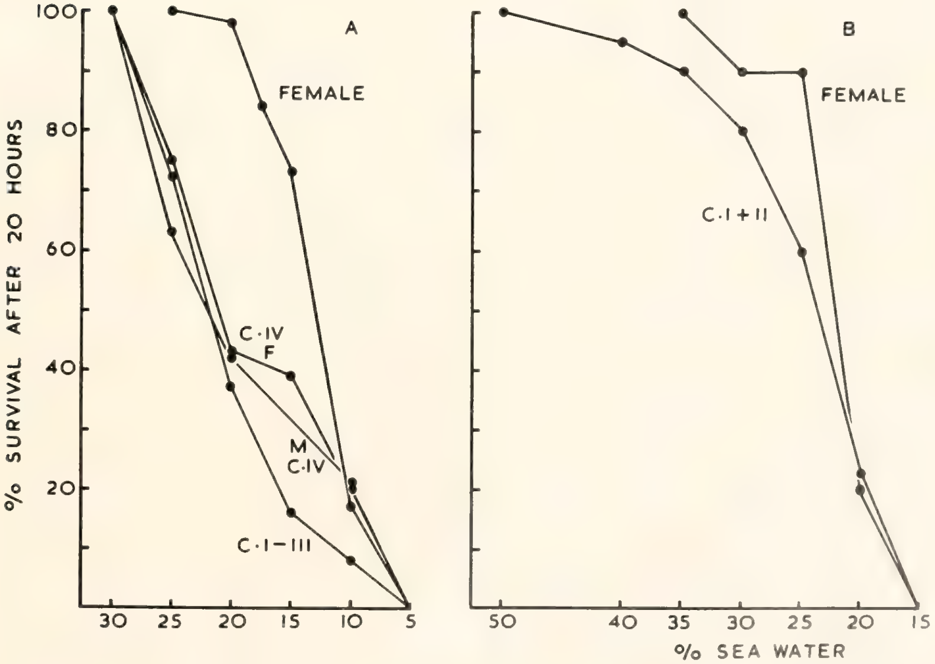


FIGURE 2. Per cent survival of copepodites and adult females after 20 hours in diluted sea water. A, *Acartia tonsa*. B, *A. bifilosa*. C, I–IV = copepodite stages; M = male; F = female. Insufficient *A. bifilosa* copepodites were available to justify analysis of the stages separately. *A. tonsa* Stage I, II, and III copepodites gave similar results and a mean survival value is plotted for each salinity.

TABLE II

Salinity tolerances of cirripedes and decapods

Species	Stage in life-history	Experimental temperature (° C.)	Range of salinities causing mortality (% sea water)	Range of lethal salinities (% sea water)
Elminius modestus	Stage VI nauplius	16.2	0–35	0–30
Carcinus maenas	Stage I zoea	17.0	0–90	0–35
	Megalopa	17.0	0–45	0–27.5
Porcellana longicornis	Stage I zoea	17.0	0–65	0–40
	Stage II zoea	17.0	0–65	0–40
	Post larva	17.0	0–70	0–57.5

ent, however, if survival after 20 hours is assessed (Fig. 2). With *A. tonsa* (Fig. 2 A), survival of adult females exceeded that of all larval stages in dilutions ranging from 15% to 25% sea water; in 10% sea water, survival was similar to that of Stage IV copepodites only, implying that these copepodites were less sensitive to dilution than the younger larvae. With *A. bifilosa* (Fig. 2 B), Stage I + II copepodites were slightly less tolerant than females in certain dilutions.

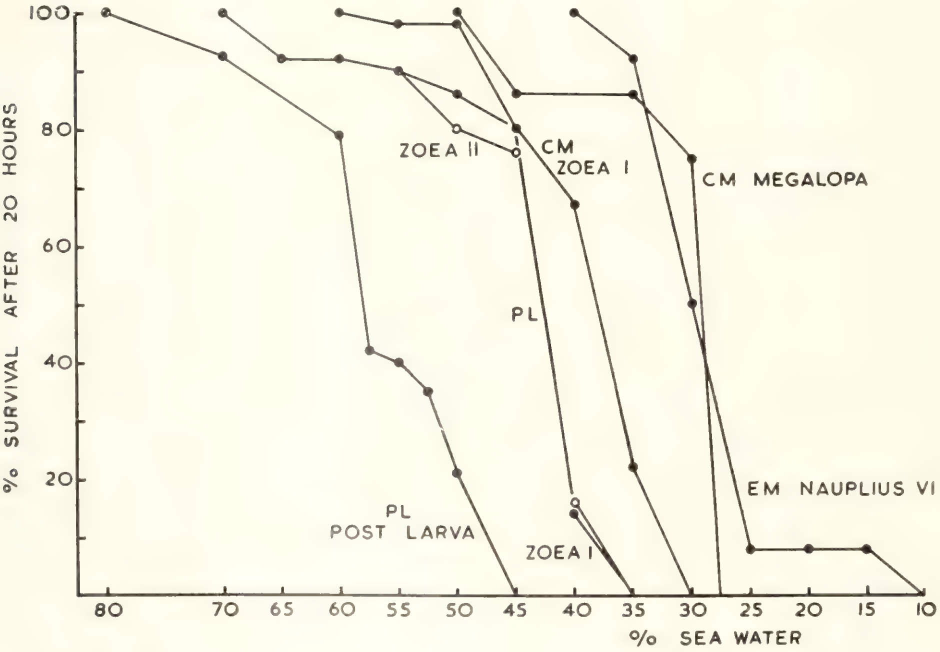


FIGURE 3. Per cent survival of decapod and cirripede developmental stages after 20 hours in diluted sea water. CM = *Carcinus maenas*; EM = *Elminius modestus*; PL = *Porcellana longicornis.*

Developmental stages of decapods

Table II presents data obtained from *Carcinus* Stage I zoeae and megalopa post larvae. The experiments using the megalopas are preliminary, as only a few animals were available. The zoeae were less resistant to dilution than the older megalopas. Figure 3, showing survival after 20 hours, demonstrates the greater susceptibility of the zoeae to water of lowered salinity. All megalopas surviving at salinities greater than 30% sea water moulted into crabs. Some megalopas moulted successfully in 30% sea water but others died while attempting to metamorphose; no moults occurred in the lethal salinities.

Results are included in Table II for *Porcellana*. It appears that Stage I and II zoeae had similar salinity tolerances and that they were more resistant to dilution than older post larval stages. This trend is obvious in Figure 3. In a further experiment lasting for two days, Stage II zoeae which were ready to moult into post larvae were used. Fewer moults occurred in 70% sea water than in media of higher salinity and none took place in 65% sea water or below.

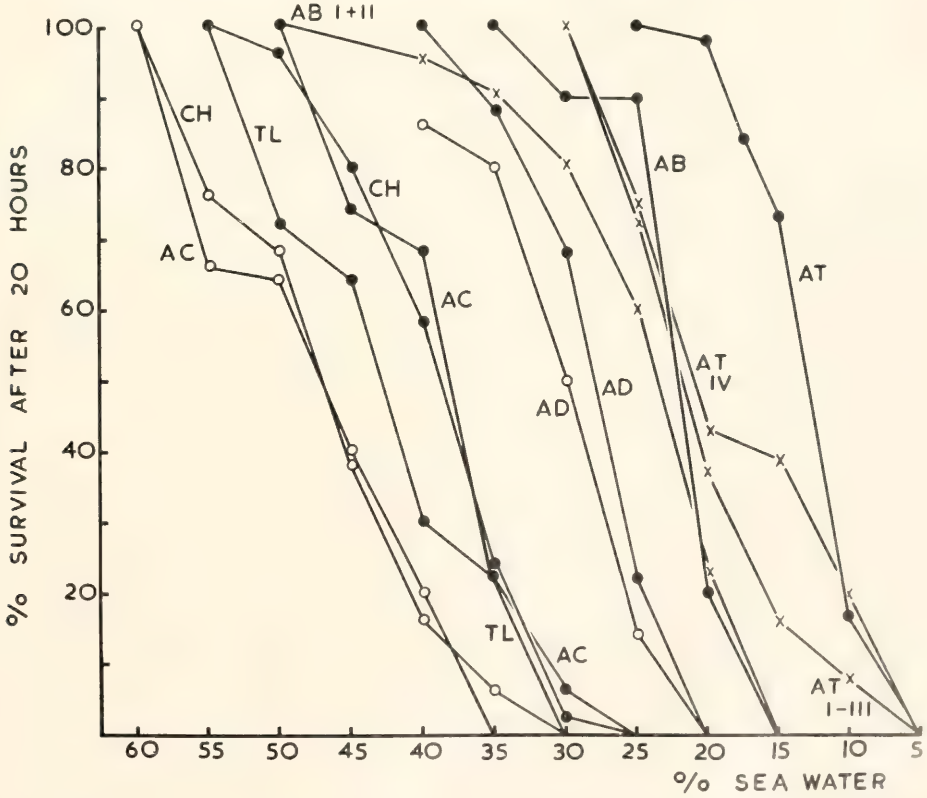


FIGURE 4. Per cent survival of copepods after 20 hours in diluted sea water. AB = *Acartia bifilosa;* AC = *A. clausi;* AD = *A. discaudata;* AT = *A. tonsa;* CH = *Centropages hamatus;* TL = *Temora longicornis;* black circle = adult females; white circle = adult males; cross = copepodites, Stage I, II, III, or female IV.

Larvae of cirripedes

Tests were confined to Stage VI nauplii of *Elminius*. Mortality occurred at salinities below 40% sea water and a range of 0–30% sea water was lethal (Table II, Fig. 3). During the experiment, many nauplii developed into cyprids. Thus, at salinities ranging from 100% to 50% sea water, the number of larvae moulting successfully into cyprids during the initial 20 hours varied between 24% and 46%. After 5 days at 100–55% sea water, 96–100% had moulted, as against 84% for 50% sea water. In 45% sea water, only 12% moulted during the first 20 hours but the number rose to 80% after 5 days. The nauplii in the remaining dilutions tended to swell and become immobile. Moulting was further delayed in both 40% and 35% sea water, and although all the nauplii in a range of 40–50% sea water had eventually become cyprids within 9 days, only 85% of the nauplii surviving in 35% sea water metamorphosed. No moults occurred at the lethal salinities. All the cyprids except for some of those in 40% and 35% sea water survived over a 7-day period following their initial appearance.

Comparison of salinity tolerances

Results obtained for all crustaceans, including those already discussed, can be compared in Figures 3 and 4 which show survival after exposure to diluted sea water for 20 hours. Relevant data are presented in Table III, where the different groups of animals have been arranged in a definite order according to their salinity tolerances. This order has been assessed by collating the 20-hour graphs and the data in Table III. Those animals with the greatest resistance to dilution are placed at the top of the list and those with the poorest salinity tolerances are assigned to the bottom.

TABLE III

Salinity tolerances of zooplankton taken from Southampton Water

Species	Stage in life-history	Field temperature at time of collection (° C.)	Experimental temperature (° C.)	Duration of expt. (days)	Range of salinities causing mortality		Range of lethal salinities	
					(% sea water)	(‰*)	(% sea water)	(‰*)
Acartia tonsa	adult female†	19.7	20.0	8	0–15	0–5.4	0–10	0–3.8
Acartia tonsa	adult female†	19.7	17.0	8	0–25	0–9.1	0–10	0–3.8
Acartia tonsa	Stage I–IV copepodite	19.7	17.0	5	0–25	0–9.1	0–20	0–7.4
Acartia bifilosa	adult female†	8.1	10.0	9	0–40	0–15.1	0–20	0–7.8
Acartia bifilosa	Stage I–III copepodite	8.1	10.0	3	0–40	0–15.1	0–20	0–7.8
Acartia discaudata	adult female†	15.3	16.0	5½	0–35	0–12.7	0–25	0–9.3
Elminius modestus	Stage VI nauplius	17.2	16.2	5	0–35	0–12.3	0–30	0–10.3
Acartia discaudata	adult male	15.3	16.0	5½	0–40	0–14.8	0–30	0–11.2
Carcinus maenas	megalopa	17.0	17.0	14	0–45	0–15.9	0–27.5	0–9.9
Acartia clausi	adult female	16.1	15.2	3	0–45	0–15.8	0–35	0–12.5
Centropages hamatus	adult female	15.0	13.5	8½	0–55	0–20.2	0–35	0–13.1
Carcinus maenas	Stage I zoea	17.0	17.0	7	0–90	0–33.0	0–35	0–12.5
Temora longicornis	adult female	15.3	14.1	5½	0–50	0–18.1	0–40	0–14.7
Porcellana longicornis	Stage I–II zoea	18.3	17.0	3½	0–65	0–23.9	0–40	0–14.8
Centropages hamatus	adult male	15.0	13.5	8½	0–55	0–20.2	0–45	0–16.3
Acartia clausi	adult male	16.1	15.2	3	0–55	0–19.2	0–45	0–15.8
Porcellana longicornis	post larva	**	17.0	6	0–70	0–25.6	0–57.5	0–21.0

† Results published in 1963.
* Salinity value determined by titration
** Reared from Stage II zoeae and used 12 hours after appearance.

DISCUSSION

The experiments on *Acartia* and *Centropages* indicate that adult males are less tolerant of dilution than the females. A survey of zooplankton changes occurring in Long Island Sound has led Conover (1956) to suggest that unfavorable conditions probably affect the male *Acartia* first and his field evidence is therefore particularly relevant. As the survival of copepods in diluted sea water partly depends on the sex, it is not surprising that the influence of dilution on swimming behavior also differs between males and females (Lance, 1962). Thus, it is the males which show the most marked changes in vertical distribution when the salinity of sea water is lowered.

The experimental evidence suggests that copepodites of *Acartia* are slightly less resistant to dilution than adult females but these results should, however, be accepted with caution. The larvae did not thrive in full-strength sea water and those which attempted to moult failed to complete development, whereas the adults in general survived well. Hence the poorer salinity tolerances of the copepodites could be associated with general unhealthiness in the laboratory. The seasonal study of the development of *Acartia* conducted by Conover (1956) does, however, support the above experimental findings. Conover found that the young developmental stages began to disappear somewhat earlier than the adults, and he concluded that unfavorable changes in the environment were affecting the young stages sooner than the adult females.

Variation in salinity tolerance may also exist between the various developmental stages of a species. Thus, the mortality of Stage IV copepodites and decapod zoeae in diluted sea water differed from that of earlier copepodites and post larvae, respectively. It is interesting that differences have been recorded between the zoeal stages of both *Sesarma* and *Panopeus* crabs, and that once the megalopa is reached, a wider range of salinities is tolerated (Costlow *et al.*, 1960, 1962).

The development of *Carcinus* megalopas, *Porcellana* zoeae, and *Elminius* nauplii was delayed or prevented at the lowest salinities. Such findings endorse existing information. Thus, during attempts to rear the crabs, *Sesarma, Panopeus,* and *Hepatus* in diluted sea water, it is found that moulting can be delayed, development can be slower, and the duration of individual stages can be longer; at the lowest salinities, complete development to the crab stage does not take place (Costlow and Bookhout, 1959, 1962; Costlow, 1960; Costlow *et al.*, 1960, 1962). The quiescence and delayed development displayed by *Elminius* nauplii in diluted sea water can also occur amongst larvae of the barnacle *Balanus* (Barnes, 1953). Many planktonic crustaceans pass through several moults before becoming adults and it is probable that sensitivity to dilution will vary according to whether an individual is at a pre-moult, inter-moult or post-moult phase.

It is obvious that the range of salinity compatible with life can vary not only between the different developmental stages of a single species but also between one species and another. In compiling the list of salinity tolerances for zooplankton from Southampton Water, it has not been practicable to conduct exhaustive acclimation tests and therefore the ultimate survival limits controlled by the genotype (see Prosser, 1955, 1957, 1958) cannot be predicted. Even so, the experimental data do aid the interpretation of field records.

Elminius nauplii showed no mortality in dilutions above 12.3‰ whereas Barnes (1953) has deduced from experiments on barnacle nauplii that *Balanus balanoides*, *B. crenatus* and *B. balanus* are unlikely to spread into waters with salinities below 10‰. The ability of *Elminius* to withstand greater dilution could be an important factor in competition between the species, especially as a high degree of eurythermy has been attributed to *Elminius* (Crisp and Davis, 1955).

The salinity tolerances determined in the laboratory for *Acartia tonsa*, *A. bifilosa* and *A. discaudata* are in general agreement with field observations on geographical distribution (Lance, 1963). The field records for these three species were reviewed in 1963 but the additional species studied in this present paper still have to be considered:

Acartia clausi occurs around the British Isles not only offshore but also in bays and harbors of low salinity (Gurney, 1931; Wells, 1938; Conover, 1957; Raymont and Carrie, 1958, 1959). Although *A. clausi* can be plentiful at salinities as low as 18.4‰ (Farran, 1910) and withstands abrupt changes of 9–29.9‰ (Deevey, 1948), it is less tolerant of dilution than *A. tonsa* and less able to propagate in low-salinity waters (Jeffries, 1962). The distribution of *A. clausi* is world-wide, yet it appears to be the least tolerant of the four *Acartia* species.

Centropages hamatus is more common in coastal waters than in the open sea. It has been recorded at a salinity range of 23.9–13.5‰ (De Lint, 1922, cited in Gurney, 1931) and penetrates into estuarine regions around the British Isles (Gurney, 1907, 1931; Rees, 1938; Wells, 1938; Conover, 1957). This species is considered to be more neritic than *Temora* (Hansen, 1960).

Temora longicornis only penetrates estuaries where there is an active renewal of water from outside (Bigelow and Sears, 1939) and high oceanic salinities may be effective barriers to distribution (Bigelow, 1926). It occurs in British estuaries (Wells, 1938; Conover, 1957; Marine Biological Association, 1957) and has been found elsewhere at ranges of 25–31‰ (Deevey, 1948) and 6–35‰ (Bigelow, 1926).

The following order of salinity tolerance is based on experimental results: *Acartia tonsa* > *A. bifilosa* > *A. discaudata* > *A. clausi* > *Centropages hamatus* > *Temora longicornis*. This order of tolerance is also indicated by existing field observations on the extent to which different species enter and propagate in low-salinity waters. In the laboratory, *Centropages hamatus* (unlike the other species) showed poorer survival in full-strength sea water than in dilutions down to 60% sea water. Bigelow's comment (1926) that this copepod occurs chiefly in waters below 32.5‰ is therefore of special interest. The unexpected sparcity of *Centropages* in certain localities may reflect a response to different types or qualities of water (see Raymont and Miller, 1962; Provasoli, 1963).

SUMMARY

1. The salinity tolerances of copepods varied according to the stage in the life-history. Adult males were less resistant to dilution than females (*Acartia discaudata*, *A. clausi*, *Centropages hamatus*), while copepodites appeared to be slightly less tolerant than adult females (*Acartia tonsa*, *A. bifilosa*).

2. *Carcinus maenas* zoeae showed a higher mortality in diluted sea water than the megalopa stages, whereas *Porcellana longicornis* zoeae were more tolerant than the post larvae.

3. The moulting of *Carcinus* megalopas and *Porcellana* zoeae into crabs and post larvae, respectively, and the development of *Elminius modestus* nauplii into cyprids were prevented or delayed by water of low salinity.

4. The salinity tolerances of important members of the zooplankton of Southampton Water were compared.

LITERATURE CITED

Barnes, H., 1953. The effect of lowered salinity on some barnacle nauplii. *J. Animal Ecol.*, **22**: 328–330.

Barnett, P. R. O., 1959. The ecology of harpacticoids on a mudflat with special reference to *Platychelipus*. Ph.D. Thesis, University of Southampton.

Battaglia, B., and G. W. Bryan, 1964. Some aspects of ionic and osmotic regulation in *Tisbe* (Copepoda, Harpacticoida) in relation to polymorphism and geographical distribution. *J. Marine Biol. Assoc.*, **44**: 1–15.

Bigelow, H. B., 1926. Plankton of the offshore waters of the Gulf of Maine. *Bull. U. S. Bur. Fisheries,* **40**, *2*: 1–509.

Bigelow, H. B., and M. Sears, 1939. Studies of the waters of the continental shelf, Cape Cod to Chesapeake Bay. III. A volumetric study of the zooplankton. *Mem. Museum Comp. Zool. Harvard Collection*, **54**: 183–387.

Bullock, T. H., 1958. Homeostatic mechanisms in marine organisms. Pp. 199–210. *In:* A. A. Buzatti-Traverso (ed.), Perspectives in Marine Biology. Berkeley, University of California Press.

Conover, R. J., 1956. Oceanography of Long Island Sound, 1952–1954. VI. The biology of *Acartia clausi* and *A. tonsa. Bull. Bingham Oceanog. Collection,* **15**: 156–233.

Conover, R. J., 1957. Notes on the seasonal distribution of zooplankton in Southhampton Water with special reference to the genus *Acartia. Ann. Mag. Nat. Hist., Ser. 12,* **10**: 63–67.

Costlow, J. D., 1960. The effect of temperature and salinity on the development of crustacea larvae. Rep. Challenger Soc., *3,* no. 12.

Costlow, J. D., and C. G. Bookhout, 1959. The effects of salinity and temperature on larval development of Brachyura reared in the laboratory. Amer. Assoc. Adv. Sci.; Intern. Oceanog. Congr., 228–229.

Costlow, J. D., and C. G. Bookhout, 1962. The larval development of *Hepatus epheliticus* (L.) under laboratory conditions. *J. Elisha Mitchell Sci. Soc.*, **78**: 113–125.

Costlow, J. D., C. G. Bookhout and R. Monroe, 1960. The effect of salinity and temperature on larval development of *Sesarma cinereum* (Bosc) reared in the laboratory. *Biol. Bull.,* **118**: 183–202.

Costlow, J. D., C. G. Bookhout and R. Monroe, 1962. Salinity-temperature effects on the larval development of the crab, *Panopeus herbstii* Milne Edwards, reared in the laboratory. *Physiol. Zoöl.,* **35**: 79–93.

Crisp, D. J., and P. A. Davis, 1955. Observations *in vivo* on the breeding of *Elminius modestus* grown on glass slides. *J. Marine Biol. Assoc.,* **34**: 357–380.

Deevey, G. B., 1948. The zooplankton of Tisbury Great Pond. *Bull. Bingham Oceanog. Collection*, **12**: 1–44.

Eltringham, S. K., and P. R. O. Barnett, 1958. Survival at reduced salinities and osmoregulation in *Limnoria* (Isopoda) and *Platychelipus* (Harpacticoida). Rep. Challenger Soc., *3,* no. 10.

Farran, G. P., 1910. Copepoda. *Bull. Trim. Cons. Intern. Explor. Mer,* **I**: 60–105.

Gross, F., 1937. Notes on the cultures of some marine plankton organisms. *J. Marine Biol. Assoc.*, **21**: 753–768.

Gurney, R., 1907. The Crustacea of the East Norfolk rivers. *Trans. Norfolk Norw. Nat. Soc.*, **8**: 410–438.

Gurney, R., 1931. British fresh-water Copepoda. Ray Soc. Publ., I: 238 pp.

Hansen, V. K., 1960. Investigations on the quantitative and qualitative distribution of zooplankton in the southern part of the Norwegian Sea. *Med. Danmarks Fiskeri-og Havundersogelser,* **II**, Nr. 23: 1–53.

Hopper, A. F., 1960. The resistance of marine zooplankton of the Caribbean and South Atlantic to changes in salinity. *Limnol. Oceanog.*, **5**: 43–47.

Jeffries, H. P., 1962. Succession of two *Acartia* species in estuaries. *Limnol. Oceanog.*, **7**: 354–364.

Lance, J., 1960. Effects of water of reduced salinity on the zooplankton of Southampton Water. Ph.D. Thesis, University of Southampton.

Lance, J., 1962. Effects of water of reduced salinity on the vertical migration of zooplankton. *J. Marine Biol. Assoc.*, **42**: 131–154.

Lance, J., 1963. The salinity tolerance of some estuarine planktonic copepods. *Limnol. Oceanog.*, **8**: 440–449.

Marine Biological Association, 1957. Plymouth Marine Fauna.

Marshall, S. M., A. G. Nicholls and A. P. Orr, 1935. On the biology of *Calanus finmarchicus*. VI. Oxygen consumption in relation to environmental conditions. *J. Marine Biol. Assoc.*, **20**: 1–27.

Matutani, K., 1962. Studies on the temperature and salinity resistance of *Tigriopus japonicus*. IV. Heat resistance in relation to salinity of *Tigriopus japonicus* acclimated to dilute and concentrated sea waters. *Physiol. and Ecol.* (*Kyoto*), **10**: 63–67.

Prosser, C. L., 1955. Physiological variation in animals. *Biol. Rev. Cambridge Phil. Soc.*, **30**: 229–262.

Prosser, C. L., 1957. The species problem from the viewpoint of a physiologist. Publ. Amer. Assoc. Adv. Sci., no. 50: 339–369.

Prosser, C. L., 1958. General summary: the nature of physiological adaption. Pp. 167–180. *In:* C. L. Prosser (ed.), Physiological Adaption. Lord Baltimore Press, Baltimore.

Provasoli, L., 1963. Organic regulation of phytoplankton fertility. Pp. 165–219. *In:* M. N. Hill (ed.), The Sea, Vol. 2. Interscience, New York.

Ranade, M. R., 1957. Observations on the resistance of *Tigriopus fulvus* (Fischer) to changes in temperature and salinity. *J. Marine Biol. Assoc.*, **36**: 115–119.

Raymont, J. E. G., and B. G. A. Carrie, 1958. Quantitative studies on the zooplankton of Southampton Water. Rep. Challenger Soc., *3*, no. 10.

Raymont, J. E. G., and B. G. A. Carrie, 1959. The zooplankton of Southampton Water. Amer. Assoc. Adv. Sci.; Intern. Oceanog. Congr., 320–322.

Raymont, J. E. G., and R. S. Miller, 1962. Production of marine zooplankton with fertilisation in an enclosed body of sea water. *Intern. Rev. ges. Hydrobiol.*, **47**: 169–209.

Rees, C. B., 1938. The plankton in the upper reaches of the Bristol Channel. *J. Marine Biol. Assoc.*, **23**: 397–425.

Wells, A. L., 1938. Some notes on the plankton of the Thames estuary. *J. Animal Ecol.*, **7**: 105–124.

Zinn, D. J., 1942. An ecological study of the interstitial fauna of some marine sandy beaches with special reference to Copepoda. Ph.D. Dissertation, Yale University.

CYTOGENETIC EFFECTS OF CHEMOSTERILANTS IN MOSQUITOES. II. MECHANISM OF APHOLATE–INDUCED CHANGES IN FECUNDITY AND FERTILITY OF AEDES AEGYPTI (L.)

K. S. RAI

Department of Biology, University of Notre Dame, Notre Dame, Indiana[1]

The successful eradication of the screw-worm fly from the island of Curaçao and the southeastern United States through the release of males sterilized by gamma-radiation (Knipling, 1959) has stimulated great interest in the possibility of using certain chemicals for the same purpose. As a result, considerable progress has been made in this field during the last few years. The field of insect chemosterilants is expanding so rapidly that three reviews (Weidhaas and McDuffie, 1963; Smith, 1963; Smith *et al.,* 1964) have appeared almost simultaneously. However, a perusal of the literature indicates that most of the research in this area is skewed in the direction of applied prospects for insect control. Relatively small effort has been expended in studying some of the biological effects of chemosterilants or the detailed mechanisms by which they interfere with insect reproduction.

Smith *et al.* (1964) have prepared an extensive list of some of the more important chemosterilants used against a number of insect species. With mosquitoes, approximately 20 chemical compounds have been shown to affect the fertility of *Anopheles quadrimaculatus, Aedes aegypti* and *Culex tarsalis* (Weidhaas and McDuffie, 1963). However, three of them, apholate, aphamide and tepa, appear to be more promising. They have been used to sterilize both sexes of *A. quadrimaculatus* and *A. aegypti* (Weidhaas *et al.,* 1961) and *Culex quinquefasciatus* (Murray, 1963).

Some studies have been conducted to analyze the effects of chemosterilants on insect gonads. An inhibition of ovarian growth in *Drosophila melanogaster* with folic acid antagonists was reported by Goldsmith *et al.* (1948) and Goldsmith and Frank (1952). Mitlin *et al.* (1957), and Mitlin and Baroody (1958) showed similar effects in house flies treated with different mitotic poisons and tumor-inhibiting substances. Alkylating agents have been likewise shown to retard ovarian growth in house flies (LaBrecque, 1962) and screw-worm flies (Chamberlain, 1962; Crystal and LaChance, 1963). Bertram (1963) observed ovarian degeneration in *Aedes aegypti* after thiotepa treatment. Most of these studies, however, did not go into the histological basis for the decreased ovarian size. Except perhaps in *Drosophila melanogaster* (Cantwell and Henneberry, 1963) and *Musca domestica* (Morgan and LaBrecque, 1962) detailed studies of the effects of chemosterilants on insect oogenesis have not been reported.

---

[1] This work received support from the following sources: Research grants No. GM 11753 and AI 02753, National Institutes of Health, U. S. Public Health Service and the Radiation Laboratory operated by the University of Notre Dame and supported in part under Atomic Energy Contract (11-1)-38. This is AEC Document No. COO-38-353.

Present studies were undertaken to find a cytogenetic basis for apholate-induced changes in the fecundity and fertility of *Aedes aegypti.* The effects of apholate on the mitotic chromosomes in the brain cells of this species have been reported in an earlier paper (Rai, 1964). The effects of this chemical on the development of ovarian tissues are presented in this paper. Some observations on the effect of apholate on testicular tissues are also included.

MATERIALS AND METHODS

The Rock strain of *Aedes aegypti* was used in this study. Eggs of this strain were hatched in deoxygenated water. The growing larvae were reared in white enamel pans containing tap water in an incubator maintained at 80.6° F. Two days after hatching, when most of the larvae were second instars, they were hand-picked and transferred to pint containers (50 per container). Each of these contained 250 ml. solution of 15 ppm apholate in tap water. Fifty larvae per cup were kept as controls in 250 ml. untreated tap water. The larvae were fed with approximately equal amounts of dog-food pellets (Gaines) from time to time.

Treated pupae were picked, rinsed in tap water and then kept in untreated tap water until emergence. After emergence the adults were taken out of the incubator and were maintained in a rearing room with a temperature of 80° F. (±4° F.) and relative humidity of 80% (±10%).

At regular intervals, ovaries from mosquitoes of known ages were dissected in *A. aegypti* saline (Hayes, 1953). Whole-mounts of the dissected ovaries were made either in this saline solution or in 0.5% acetocarmine. Some dissections of testes from 8–9-day-old, unmated, treated and untreated males were also made.

Photomicrographs of various developmental stages were taken with a 35 mm. Zeiss Ikon camera from temporary slides. Panatomic-X, black and white film was used.

Four- to five-day-old adults from treated and untreated batches were crossed in single pairs in different combinations. Ten to 20 single-pair crosses were set up in each case and 1–3 egg batches were collected from each female after successive blood feedings. Spermathecae of several females kept with treated males were examined for the presence of motile sperms.

NORMAL OVARIAN DEVELOPMENT

The normal course of oogenesis in mosquitoes has been described in considerable detail by various investigators (Mer, 1936; Parks, 1955; Bertram, 1962). Since the present account is concerned chiefly with the effects of apholate on oogenesis in *A. aegypti,* it is necessary to describe briefly the normal ovarian development. The following stages of follicular development, described by Mer (1936) for *Anopheles elatus,* may be applied to *Aedes aegypti* also:

N: Follicle consists of 8 undifferentiated cells; cubical epithelium cells surround the spherical follicle.

I: One oocyte gets differentiated from the seven nurse cells.

I–II: Some yolk appears around the nucleus in the oocyte cytoplasm; follicle becomes oval in shape.

II: Oocyte grows by the accumulation of numerous yolk granules and occupies about half the follicle.
III: Oocyte grows to about ¾ of the follicle; oocyte nucleus obscured by the yolk.
IV: Follicle elongates and the nurse cells are restricted to a small uppermost part of it; oocyte fills almost the entire follicle.
V: Chorion appears around the egg; remains of the nurse cells are found at the proximal end of the follicle; egg floats appear and the egg is ready for laying.

Results

Effect on female fecundity

Data included in Table I indicated that treating the female larvae with 15 ppm apholate solution at second instar resulted in almost complete infecundity. Most of the females emerging from larvae so treated (hereafter designated treated females) did not oviposit. Some that did, laid only a few eggs, much fewer than any of the untreated females. Although there were some exceptions, the treated females that laid some eggs after the first blood meal were usually the ones that oviposited after subsequent blood meals also, and *vice versa.*

Table I

Effect of 15 ppm apholate on the fertility and fecundity of Aedes aegypti

Cross*		1st blood meal		2nd blood meal		3rd blood meal	
Female	Male	Eggs laid/♀	% Eggs hatched/♀	Eggs laid/♀	% Eggs hatched/♀	Eggs laid/♀	% Eggs hatched/♀
U ×	U	109(12)**	97.7	113(11)	97.6	115(9)	96.7
U ×	T	100(18)	10.0	109(14)	5.7	86.5(13)	4.4
T ×	U	7(11)	34.0	4(9)	68.2***	5(9)	29.6
T ×	T	3(17)	5.6	2(15)	18.0	1(12)	0.0

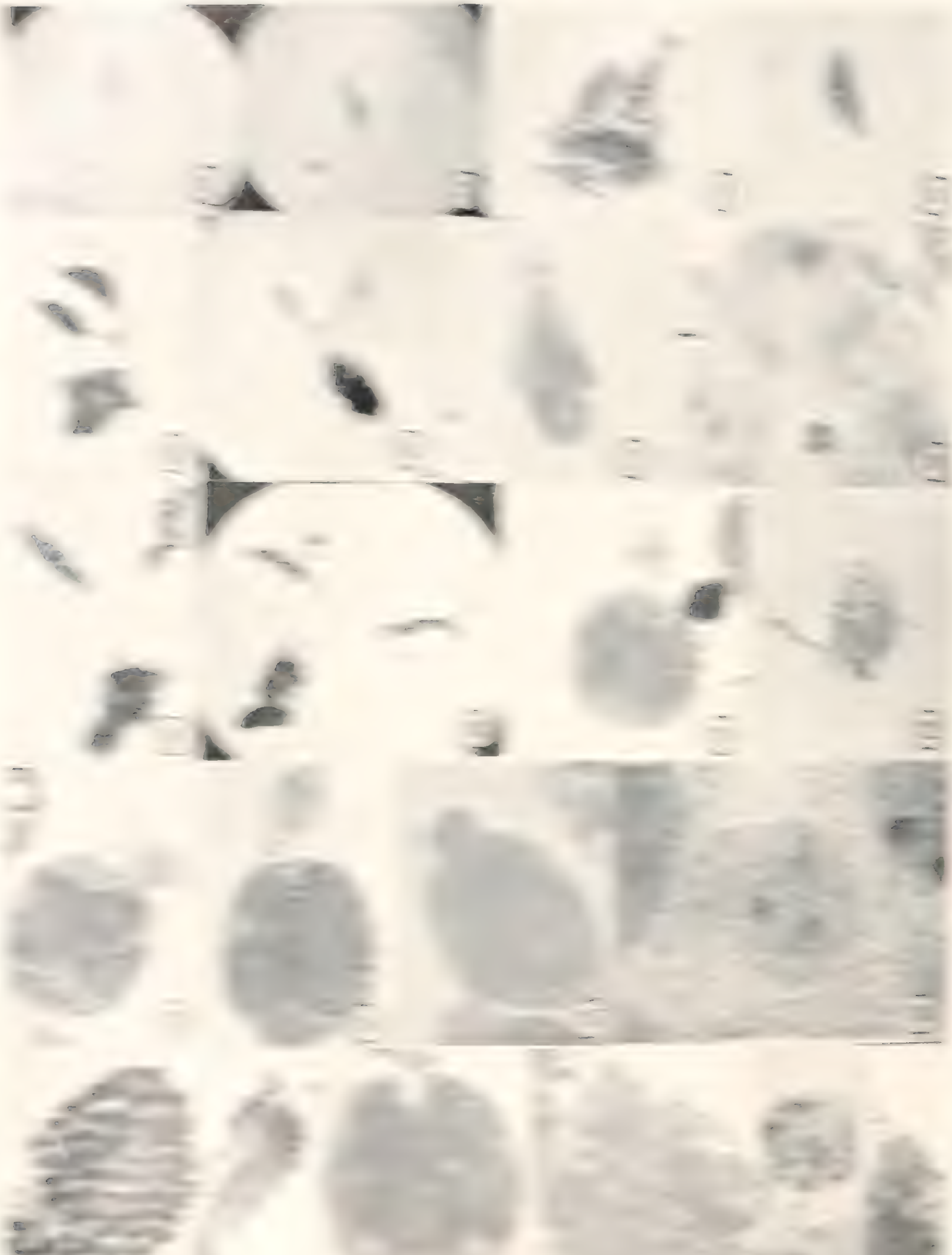
* U = untreated; T = treated.
** Figures in parentheses represent number of single pair crosses made.
*** Only one ♀ laid 39 eggs, out of which 27 hatched.

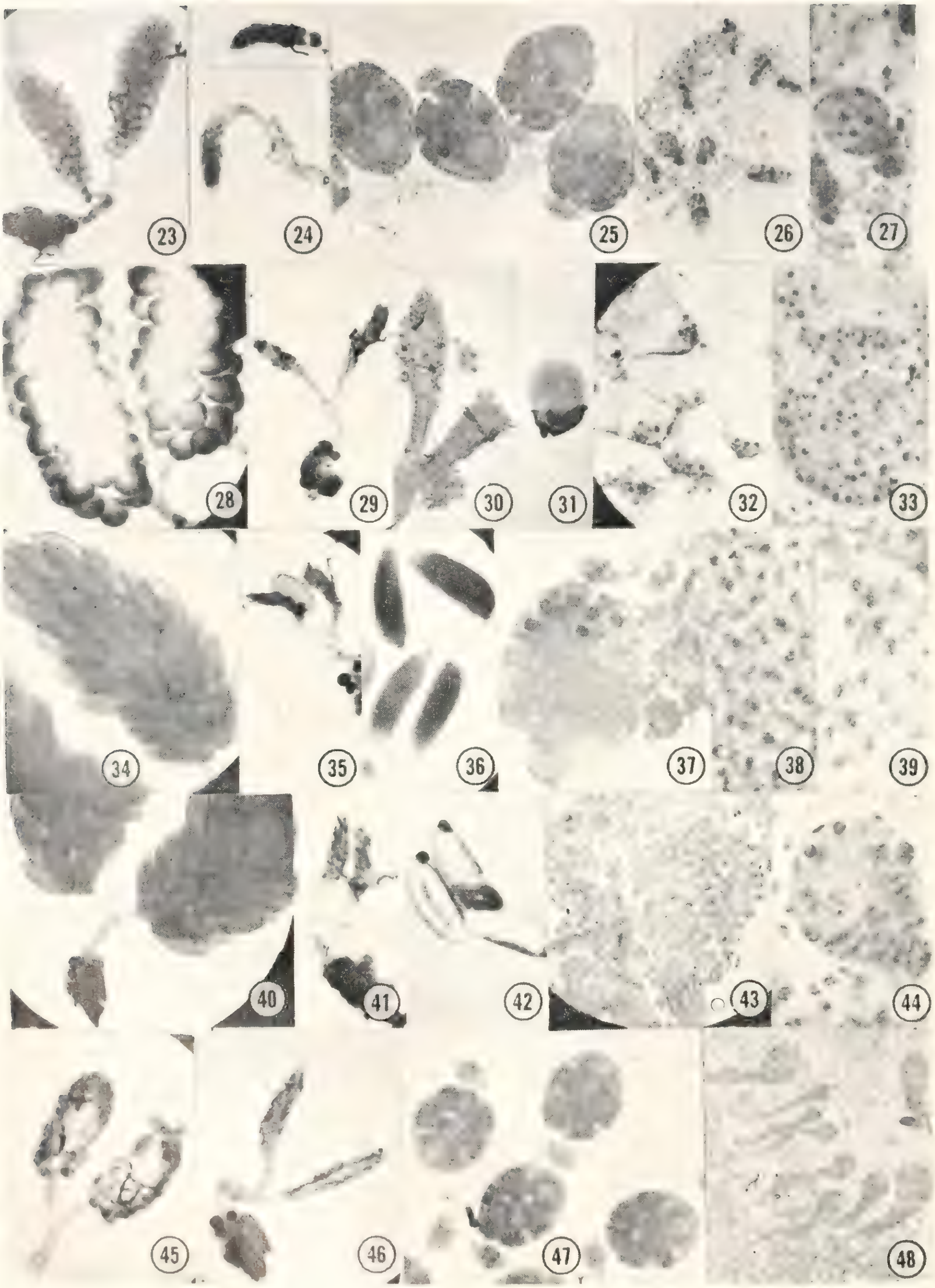
Effect on oogenesis

The details of this process in the present account will be restricted to the facts needed to evaluate properly the effects of apholate on oogenesis and to make the experimental portion of this investigation comprehensible. Results of ovarian dissections from different age groups follow. Reference made to various stages in developing follicles is based on Mer's (1936) classification.

24–28-hour-old pupae (Figs. 1–4)

There did not appear to be much difference in the size of the ovaries of the untreated (Fig. 1) and treated (Fig. 2) 24–28-hour-old pupae. The primary (first) follicle consisted of 8 undifferentiated cells with rather prominent nuclei in both the untreated (Fig. 3) and treated ovarioles (Fig. 4). This corresponds to stage "N" of Mer (1936).




23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

FIGURES 23–27 and 33 (6 hours after blood feeding). Note the difference in size of the ovaries from control (Figure 23; 20 ×) batches and those of the treated ones (Figure 24; 20 ×). The follicles in the former are at Stage II (Figure 25; 80 ×); the latter show follicular

Approximately 24 hours after emergence the ovaries and the ovarioles in the controls enter a resting stage and do not develop significantly until the female is given a blood meal.

50-hour-old adults (Figs. 13–16)

Some yolk granules were clearly visible around the nucleus in the oocyte cytoplasm towards the posterior end of the primary follicle in the untreated females (Figs. 13–15; stage I–II). The yolk granules, which appear much earlier, may occupy one third of the follicle in 50- to 74-hour-old adults.

In the treated material, nurse cells in the first follicles in some cases showed signs of degeneration, as evidenced by clumps of chromatin material. At least three such clumps are visible in Figure 16.

87–91-hour-old adults (Figs. 17–22)

Ovaries in untreated mosquitoes were considerably larger and a dissection of each disclosed a large number of uniformly developing ovarioles (Fig. 17). In the treated ovaries, however, most of the ovarioles were considerably retarded in development (Figs. 18 and 20). This retardation usually occurs to different degrees in different follicles. It may depend upon the stage of follicular development at which degeneration begins. As a result, a great variability in follicle sizes was observed. A few of them approach the size of those in the controls. These may ultimately be laid as eggs. Mostly, however, they remained small and without much nuclear differentiation. Approximately one third of the developing follicles in untreated females was occupied by yolk (Fig. 19), but the oocyte nucleus was still quite visible. The follicular epithelium in controls was well defined, but showed

---

degeneration to different degrees (Figure 26; 80 × and Figure 27; 200 ×). The disintegration of follicular epithelium is almost complete (Figure 33; 200 ×) in a treated female.

Figures 28–32 (24 hours after blood feeding). Ovaries from untreated (Figure 28; 20 ×) and treated females (Figure 29; 20 ×) differ still greatly in size. A squashed ovary from a treated female is shown in Figure 30 (20 ×). Yolk in the oocyte cytoplasm occupies most of the stage III follicle in untreated females (Figure 31; 20 ×). The nurse cells, which are pushed down towards the anterior end of the follicle, become clearly visible when a coverslip is gently put on (Figure 32; 20 ×).

Figures 34–39 (50 hours after blood feeding). Control ovaries are shown in Figure 34 (20 ×) and the treated in Figure 35 (20 ×). Note one well developed egg follicle in the latter. Follicles in the former are at stage IV (Figure 36; 20 ×), and are almost full of yolk. Figure 37 (80 ×) shows another stage IV follicle developing in a treated female. Neighboring follicles in this case are degenerated. The follicular epithelial nuclei in an untreated female are mostly in interphase (Figure 39; 200 ×), whereas most of those in a follicle from a treated female are pycnotic (Figure 38; 200 ×). Note the presence of minute granules around the walls of the latter.

Figures 40–44 (74 hours after blood meal). The follicles in control ovaries (Figure 40; 20 ×, and Figure 42; 20 ×) have developed to mature size and shape (Stage V) while the ovaries of treated mosquitoes remain much smaller (Figure 41; 20 ×). An ovarian squash shows complete follicular degeneration (Figure 43; 20 ×). One of the follicles from this squashed ovary is shown at higher magnification in Figure 44 (320 ×).

Figures 45–48 (5-day-old females). Ovaries of untreated females immediately after first oviposition are shown in Figure 45 (20 ×) and those of treated females of the same age in Figure 46 (20 ×). The latter did not oviposit. The ovarioles from control and treated ovaries are shown in Figure 47 (20 ×) and Figure 48 (80 ×), respectively.

various stages of degeneration in treated females (Fig. 21). A dumb-bell-shaped telophase bridge in a dividing nucleus of follicular epithelium is shown in Figure 22.

6 hours after blood feeding (Figs. 23–27, and 33)

Quick changes take place in ovarian development after blood feeding. Treated females take blood as eagerly as the untreated ones. The ovaries on the whole were much larger in the untreated (Fig. 23) than in the treated females (Fig. 24). The yolk around the distinct oocyte nucleus occupies approximately one half of the first follicle in the ovarioles in controls (Fig. 25; stage II). Most of the ovarioles in the treated females, however, were almost completely atrophied (Fig. 26). Oocytes were not obvious in these follicles (Fig. 27). The disintegration of follicular epithelium which set in some time earlier was almost completed in some follicles (Fig. 33).

24 hours after blood meal (Figs. 28–32)

The control ovaries continued to grow very rapidly (Fig. 28). However, the treated ones did not show any increase in size (Fig. 29). Whereas the ovarioles in a squashed ovary from a treated female were hardly visible (Fig. 30), the follicles in untreated females had greatly increased in size. This increase results from the aggregation of yolk granules in oocyte cytoplasm. The yolk occupied most of the follicle by this time, and the nurse cells were pushed down towards the anterior end of the follicle. The relative areas occupied by the yolk and the nurse cells stand out clearly when a coverslip is lightly put on the follicles (Fig. 32; Stage III).

50 hours after blood feeding (Figs. 34–39)

Ovaries from control batches are shown in Figure 34 and those from the treated ones in Figure 35. As mentioned earlier, the data in Table I indicated that some treated females laid some eggs. One such egg is shown developing in an ovary of a treated mosquito (Fig. 35) and another in another ovary (Fig. 37). The development of these occasional follicles proceeds as it would in an untreated female. The follicles in untreated females have assumed the shape of mature eggs (Fig. 36; Stage IV).

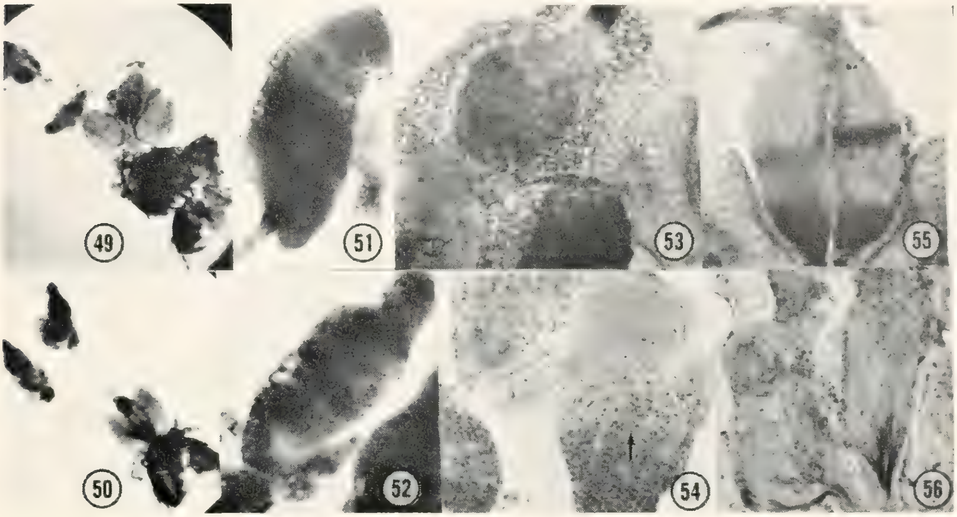
Follicular epithelial nuclei of developing eggs in the untreated series were generally in interphase (Fig. 39). In contrast, the corresponding nuclei in some of the eggs that appear to develop normally in treated mosquitoes were pycnotic and degenerated (Fig. 38). The cytoplasm, but more particularly the walls, of these cells showed the presence of minute granules not observed in the controls.

74 hours after blood feeding (Figs. 40–44)

The eggs in the untreated ovaries (Figs. 40 and 42) developed to the mature size and shape while the ovaries from the treated mosquitoes remained inhibited (Fig. 41). The ovarioles in the latter were completely inhibited in their development (Fig. 43). A typical degenerating follicle, without any epithelium, nurse cells, oocyte or yolk granules, 74 hours after blood feeding, is shown in Figure 44.

5-day-old females (Figs. 45–48)

The untreated females had laid eggs and the ovaries in these (Fig. 45) resembled those of approximately 2–3-day-old unfed females. A differentiation between the oocyte and the nurse cells was obvious in the second egg chambers (Fig. 47; Stage I–II). However, the ovaries (Fig. 46) and the ovarioles (Fig. 48) in treated females remained unchanged and degenerated.



FIGURES 49–56. Male reproductive tissues in apholate-treated and untreated males. The size of the male reproductive parts (Figure 49; 20 ×), including the testes (Figure 51; 80 ×), of 8-day-old untreated males is not markedly different from similar structures (Figure 50; 20 ×, and Figure 52; 80 ×) in 9-day-old treated males. The amount of sperm in the testes (Figure 53; 80 ×) and seminal vesicles (Figure 55; 80 ×) of the untreated males, however, appears to be more than in the testes (Figure 54; 80 ×) or in the seminal vesicles (Figure 56; 80 ×) of the treated males. The epithelium of a testis shows necrosis (Figure 54).

Effect on the male reproductive tissues (Figs. 49–56)

Dissection of the spermathecae of females caged with males developing from larvae treated at second instar (hereafter designated treated males) disclosed the presence of motile sperms. This indicated that the sperm are produced by the treated males. However, the egg hatchability data in Table I indicated that most of the sperm produced by the treated males must contain dominant lethal mutations, for when these sperms inseminated the normal females, the hatchability of the eggs was extremely low.

Several dissections of the male reproductive tissues from 8-day-old untreated and 9-day-old treated unmated, males were made. Preliminary results indicated that the size of the male reproductive structures, including the testis, was not appreciably different in the untreated (Figs. 49 and 51) and the treated (Figs. 50 and 52) males. However, the amount of sperm masses in the control testes (Fig. 53) and in the seminal vesicles (Fig. 55) often appeared to be greater than that in the same structures (Figs. 54 and 56) in the treated males. Also, the basal portion of the testis frequently contained spermatids rather than sperms as was the case in control testes. Besides, necrosis of the epithelium of the treated testes was observed (Fig. 54). These results are in agreement with those of Cantwell and Henneberry (1963) with *Drosophila melanogaster*.

DISCUSSION

It is obvious that rearing *Aedes aegypti* from second instar larvae until pupation in a solution of 15 ppm apholate, a commonly used alkylating agent, results in almost complete female infecundity. This effect is irreversible at least up to a month after emergence (three gonotrophic cycles). Bertram's (1963) data based on adult treatment indicated the same over a period of 6 weeks. The decreased female fecundity results from chemosterilant-induced interference with the normal course of oogenesis.

Two processes appear to be responsible for this. The young follicles are inhibited in their growth and differentiation. There is a considerable evidence that alkylating agents interfere with normal synthesis of the genetic material. Alexander (1960) has provided excellent insights into the mode of action of some of the alkylating agents. He has pointed out that the molecules of proteins, nucleic acids and different vitamins etc., have many sites to which the alkylating agents can and do attach. The sulfhydryl group (SH), the amino group (NH_2) and the acid group (COOH) are the main chemical groups with which alkylating agents react in living cells. DNA molecules may be joined together by alkylating agents in "unnatural configurations" (Alexander, p. 105). In a chromosome, if the two chromatids were cross-linked by an alkylating agent before the division of the cell, "the two could not separate properly during division and abnormalities would result" (Alexander, p. 104). This could easily lead to mitotic death. An active synthesis of chromatic material, which the nurse cells undergo during their endomitotic replications, will be thus greatly impeded. In the screw-worm fly it has been demonstrated that the greatest inhibition of ovarian growth occurs when the chemosterilants were applied during the endomitotic phase of the nurse cells (Crystal and LaChance, 1963). Also, as a direct or indirect consequence, yolk syntheses may never start in most of these follicles. It is well known that the synthesis of yolk in the oocyte cytoplasm does not start until the follicle has developed to a particular point. Besides this inhibition of follicular growth and differentiation, apholate brings about a degeneration of the developing follicles as well. This degeneration, which usually starts quite early in development, may initiate in the follicular epithelium and proceed inwards. Alternatively, the follicular degeneration also could lead to the collapse of the epithelial cells. Parks (1955) favored the former hypothesis for follicular degeneration in untreated normal females. In any case a complete breakdown of the ovarioles ensues. Cantwell and Henneberry (1963) have reported a similar degeneration of the ovarian tissues in *Drosophila melanogaster* after apholate treatment of adult flies.

Follicular degeneration is not an uncommon phenomenon in untreated, normal mosquitoes. However, in these it has been often ascribed to the ingestion of inadequate amounts of blood (Detinova, 1962) by the female. In the present study, this almost certainly was not the case.

It is interesting that in the treated females a few eggs, sometimes only one, may develop. This may be due to a number of causes. Certain follicles, whose threshold of sensitivity to chemical treatment may be lowest, might begin to develop normally. There may be critical stages in follicular development which if unaffected could permit growth and differentiation to continue until maturity. Bertram (1963, p. 333) in *Anopheles gambiae gambiae* has "some indication of a phase in early formation of the first follicles which is particularly susceptible to the action of the alkylating agent." It seems possible that a follicle beyond this stage may proceed with uninterrupted development. Furthermore, according to Detinova (1962, p. 42), follicular degeneration in normal females "is a supplementary regulatory process." In treated females this may enable some of the follicles to complete development.

A rather low hatchability of the few eggs laid by treated females inseminated by normal males (as compared with eggs from normal ♀ × normal ♂ crosses) indicates that these eggs must carry induced dominant lethals.

The effect of apholate on male reproductive tissues may be slightly different in certain regards from that on female tissues. As reported by other investigators also, the motile sperms are produced in the testes after treatment of immature stages, a treatment which in females almost stops all egg production. Furthermore, as is well known, the sperms from treated males are transferred to female spermathecae. Whereas inhibition of ovarian growth after chemosterilant treatment has been observed with a variety of different insects, results on testicular development vary considerably. Testes in adult screw-worms, treated with apholate at the larval stage, were smaller than normal size (Chamberlain, 1962). Murray (1963) got a similar effect with apholate in *Culex quinquefasciatus*. However, no marked decrease in the size of the testes in treated males was observed 6–7 days after apholate treatment in *Drosophila melanogaster* (Cantwell and Henneberry, 1963) or 9 days after treatment in the present study. It must be emphasized, however, that my results with testicular development are only preliminary at this stage.

There may be a reduction in the fecundity of males resulting from apholate treatment. This is indicated by the presence of fewer sperms in the anterior portion and mostly spermatids in the posterior portion of the testes of several treated males. Studies in sperm depletion by mating treated males to successive females would be much more definitive in this regard.

Most of the sperms produced by the treated males undoubtedly carried apholate-induced dominant lethals. This is evidenced by the failure of the eggs of untreated mothers to hatch after insemination by the treated males (Table I). It is generally believed that dominant lethals result from the induction of drastic chromosomal aberrations in the gametes. Rai (1964) has shown that apholate induces a large number of such aberrations in the brain cells of *Aedes aegypti*. It can be argued that if similar chromosomal changes are induced during gametogenesis also (there is evidence that they are), genetic disturbances would ensue in the fertilized egg. Mitotic anomalies and the resulting genetic imbalance which would result from the presence of, for example, large deletions, acentric or dicentric chromosomes or polyploid complements in a developing embryo would result in its death before embryogenesis is completed. Such fertilized eggs would not hatch. Fahmy and Fahmy (1954) have demonstrated that the failure of eggs to hatch after fertilization by chemically treated sperms in *Drosophila melanogaster* is due to disturbed cleavage resulting from "non-eucentric chromosomal configurations." The conclusion that dominant lethality in *Aedes aegypti* results from similar mechanisms seems quite valid.

Certain limitations of the use of chemosterilants for insect control may be pointed out. Bertram (1963) has shown that treated males of *Aedes aegypti* three weeks or so after chemosterilization appear to recover from the effects and start progressively producing more normal sperms. This, as pointed out by Bertram (p. 335), may "prejudice the success of control operations based on male chemosterilization."

Field experiments with *Anopheles quadrimaculatus* (Weidhaas *et al.*, 1962) and *Aedes aegypti* (Morgan *et al.*, 1962) are not very encouraging. Studies with *A. quadrimaculatus* "in which chemosterilants were used as a biological tool, have indicated that the laboratory colony differs from the wild population in behaviour characteristics and this difference may account for the ineffectiveness of the release of males" (Weidhaas and McDuffie, 1963, p. 269).

A great deal of basic work on the diverse effects of chemosterilants on the living systems, and on the biology and behavior of the insects in natural populations must be undertaken before a proper evaluation of the potentialities of chemosterilants can be accomplished.

The author wishes to express his thanks to his colleague, Dr. Harvey A. Bender, for critical reading of the manuscript. My thanks are also due to Dr. Samuel S. Ristich of The Squibb Institute for Medical Research, New Brunswick, N. J., for providing samples of apholate used in this study, and to Mrs. Marion Ossmann for technical assistance.

SUMMARY

1. Two-day-old larvae of *Aedes aegypti* were reared until pupation in 15 ppm apholate, a commonly used insect chemosterilant. This concentration induced almost complete female infecundity. Ovarian dissections of developing females at regular time intervals were made in order to study the underlying basis of reduced fecundity. Apholate greatly inhibited ovarian development. The follicles in treated females remained small and sooner or later underwent complete degeneration. In some of these follicles a distinction into nurse cells and oocytes never took place. In others, a complete breakdown of the follicular epithelium, nurse cells and oocyte was observed. Rarely, one or more follicles completed development in treated females. Many of these failed to hatch after insemination with normal males. This indicates the induction of dominant lethals in these eggs.

2. Motile sperms were produced by males emerging from larvae treated at second instar stage. The size of the testes in 9-day-old treated males was not greatly different from those of 8-day-old untreated males. Males used in these dissections were not mated with any females. Although conclusive data are not yet available, a lower fecundity in treated males than that of untreated males was indicated. Epithelium of the testes in some treated males showed necrosis. The so-called "male sterility" must result from induction of dominant lethality in motile sperms.

3. Some explanations concerning the mode of action of apholate and the nature of dominant lethals are discussed.

LITERATURE CITED

Alexander, P., 1960. Radiation imitating chemicals. *Sci. Amer.*, **202**: 99–108.

Bertram, D. S., 1962. The ovary and ovarioles of mosquitoes (in Age-grouping methods in *Diptera* of medical importance). Wld. Hlth. Org. Monograph no. 47: 195–204.

Bertram, D. S., 1963. Observations on the chemosterilant effect of an alkylating agent, thiotepa, on wild-caught *Anopheles gambiae* var. *melas* (Theo.) in Gambia, West Africa and on laboratory bred *A. g. gambiae* Giles and *Aedes aegypti* (L.). *Trans. Roy. Soc. Trop. Med. Hyg.*, **57**: 322–335.

Cantwell, G. E., and T. J. Henneberry, 1963. The effects of gamma radiation and apholate on the reproductive tissues of *Drosophila melanogaster* Meigen. *J. Insect Path.*, **5**: 251–264.

Chamberlain, W. F., 1962. Chemical sterilization of the screw-worm. *J. Econ. Entomol.*, **55**: 626–628.

Crystal, M. M., and L. E. LaChance, 1963. The modification of reproduction in insects treated with alkylating agents. I. Inhibition of ovarian growth and egg production and hatchability. *Biol. Bull.*, **125**: 270–277.

Detinova, T. S., 1962. Age-grouping methods in Diptera of medical importance. Wld. Hlth. Org. Monograph no. 47, 217 pp.

Fahmy, O. G., and M. J. Fahmy, 1954. Cytogenetic analysis of carcinogens and tumor inhibitors in *Drosophila melanogaster*. II. The mechanism of induction of dominant lethals by 2:4:6-tri (ethyleneimino)-1:3:5-Triazine. *J. Genet.*, **52**: 603–619.

Goldsmith, E. D., and I. Frank, 1952. Sterility in the female fruit fly, *Drosophila melanogaster*, produced by the feeding of a folic acid antagonist. *Amer. J. Physiol.*, **171**: 726–727.

Goldsmith, E. D., E. B. Tobias and M. H. Harnly, 1948. Folic acid antagonists and the development of *Drosophila melanogaster*. *Anat. Rec.*, **101**: 93.

Hayes, R. O., 1953. Determination of a physiological saline solution for *Aedes aegypti* (L.). *J. Econ. Entomol.*, **46**: 624–627.

Knipling, E. F., 1959. Sterile-male method of population control. *Science*, **130**: 902–904.

LaBrecque, G. C., 1962. The effect of apholate on the ovarian development of house flies. *J. Econ. Entomol.*, **55**: 626–628.

Mer, G. G., 1936. Experimental study on the development of the ovary in *Anopheles elatus*, Edw. (Dipt. Culic.). *Bull. Entomol. Res.*, **27**: 351–359.

Mitlin, N., and A. M. Baroody, 1958. Use of the housefly as a screening agent for tumor-inhibiting agents. *Cancer Research*, **18**: 705–710.

Mitlin, N., B. A. Butt and T. J. Shortino, 1957. Effect of mitotic poisons on house fly oviposition. *Physiol. Zoöl.*, **30**: 133–136.

Morgan, P. B., and G. C. LaBrecque, 1962. The effect of apholate on the ovarian development of houseflies. *J. Econ. Entomol.*, **55**: 626–628.

Morgan, P. B., E. M. McCray, Jr. and J. W. Kilpatrick, 1962. Field tests with sexually sterile males for control of *Aedes aegypti*. *Mosq. News*, **22**: 295–300.

Murray, W. S., 1963. The effect of apholate on the mosquito *Culex pipiens quinquefasciatus* Say. *Bull. Entomol. Soc. Amer.*, **9**: 173.

Parks, J. J., 1955. An anatomical and histological study of the female reproductive system and follicular development in *Aedes aegypti* (L.). M.S. Thesis, University of Minnesota, Minneapolis, Minn., 71 pp.

Rai, K. S., 1964. Cytogenetic effects of chemosterilants in mosquitoes. I. Apholate-induced aberrations in the somatic chromosomes of *Aedes aegypti*. *Cytologia*, **29** (In press).

Smith, C. N., 1963. Prospects for vector control through sterilization procedures (in *Vector Control*). *Supp. Bull. Wld. Hlth. Org.*, **29**: 99–106.

Smith, C. N., G. C. LaBrecque and A. B. Borkovec, 1964. Insect chemosterilants. *Ann. Rev. Entomol.*, **9**: 269–284.

Weidhaas, D. E., and W. C. McDuffie, 1963. Highlights of recent research on chemosterilants for the control of insects of medical and veterinary importance. *Bull. Entomol. Soc. Amer.*, **9**: 268–272.

Weidhaas, D. E., C. H. Schmidt and E. L. Seabrook, 1962. Field studies on the release of sterile males for the control of *Anopheles quadrimaculatus*. *Mosq. News*, **22**: 283–291.

Weidhaas, D. E., H. R. Ford, J. B. Graham and C. N. Smith, 1961. Preliminary observations on chemosterilization of mosquitoes. *N. J. Mosq. Extermin. Assoc. Proc.*, **48**: 106–109.

ON SOME PROPERTIES OF THE JELLY COAT IN OOCYTES AND MATURE EGGS OF SEA URCHINS. A STUDY OF PHASE-DEPENDENT CHANGES OF METAPLASMIC LAYERS IN THE CELL SURFACE

J. RUNNSTRÖM

Wenner-Gren Institute, Norrtullsgatan 16, Stockholm VA, Sweden

The maturation of the oocyte of the sea urchin involves a number of complicated processes. The displacement of the cortical granules is one of them (Monné and Hårde, 1951). The whole cytoplasm of the oocyte incorporates $^{35}SO_4$; during the course of maturation the labelled material is displaced toward the cortex (Immers, 1961a). For many years this writer has observed that there are considerable differences between the jelly coats of oocytes and maturing eggs in comparison with those of mature eggs. In the writer's studies, these differences revealed themselves in the way of interaction between sperm and the jelly coat of the egg. A brief survey will be given of some of the work that has been done concerning these interactions.

Hartmann and Schartau (1939) (see also Hartmann, 1956) demonstrated that a concentrated sperm suspension is able to dissolve the jelly coat of *Arbacia lixula*. They showed that the sperm liquor has a similar effect.

Vasseur (1951) also used living sperm but extracted the substance of the jelly coat of the egg by means of acid sea water (pH 5.2). The changes in viscosity of the jelly solution were measured after addition of sperm. They cause an increase in viscosity of the jelly solution. If, however, some octylalcohol is added, either before or different times after insemination, there is a sudden considerable drop in viscosity of the jelly that is not observed upon addition of octylalcohol alone. According to Vasseur the octylalcohol removes certain substances that are released when bringing the jelly coat in solution. These substances should inhibit an enzyme contained in the sperm. According to Vasseur these inhibitory substances are masked in the intact jelly; therefore a splitting of the intact jelly coat occurs upon penetration of spermatozoa. In some other investigations the splitting action of sperm extracts was tested on jelly solutions or on hyaluronate. Monroy and Ruffo (1947) and Lundblad and Monroy (1950) found a weak action on hyaluronidate but this mucopolysaccharide is not present in the jelly. A viscosity-lowering effect on jelly coat solution by sperm extract on its own species was described. The sperm extract was shown to have a proteolytic activity which may split the protein moiety of the jelly. Sensitivity of the active principle to boiling pointed to an enzyme effect. A precipitation of the jelly by basic substances present in the sperm extract may also have played a role in the investigation of Lundblad and Monroy (1950). Hultin and Lundblad (1952) demonstrated the presence of some carbohydrases in sperm extract. Hultin *et al.* (1952) introduced a method of testing the removal of the jelly coat from the eggs of *Arbacia lixula* (Naples). The spermatozoa were spun down at 6000–7000 *g* for 5 minutes and the supernatant (S1) was removed. The spermatozoa were resuspended in sea water and the centrifugation repeated. The supernatant (S2) was removed. Usually five supernatants were prepared in this way (S1-S5). The supernatants were added to an egg suspension in sea water and the sedimentation of the eggs was observed in concave dishes (Boveri dishes). The sedimenting eggs were collected in the center of the dish and it was easy to decide macroscopically as to whether the eggs had lost their jelly coats or not. These were lost by the action of S1 and S2, but later the dissolution was replaced by a precipitation of the jelly coats. This was caused by the extraction of a "jelly-precipitating factor" from the sperm (sperm antifertilizin). Hultin *et al.* proposed the abbreviation "Je Ppt F (Sp)" for this factor, "Je Ppt F(E)" being the corresponding factor from the egg. The jelly-dissolving factor from sperm was inactivated by heating to 60° for 30 minutes. This speaks in favor of its enzyme character. After heat treatment of the sperm extracts the jelly-precipitating factor became more active. This shows that the jelly-dissolving and the jelly-precipitating factor exist beside each other in perhaps all the supernatants S1-S5, but, depending on concentrations, they weaken or exclude each other. Hartmann and coworkers (see Hartmann, 1956) considered that the jelly-dissolving and -precipitating effect was due to the sperm factor "Gynogamon 2." The results reported indicate that this gamone must be separated into two factors.

Owing to outside circumstances the investigations of Hultin *et al.* concerning the jelly-dissolving factor could not be further elaborated but the results obtained by Hathaway *et al.* (1960) and Hathaway and Warren (1961) are rather analogous. Substances may be released from the surface of sperm of *Arbacia punctulata* (Woods Hole) by treatment with homologous jelly substance "fertilizin" or with 10^{-4} M sodium lauryl sulfate. These extracts caused a complete disappearance of the jelly coats surrounding the *Arbacia* eggs. The activity is destroyed by heating for 5 minutes to 80°. This corresponds well with the degree of thermolability found for the jelly-dissolving factor studied by Hultin *et al.* In the experiments of Hathaway *et al.*, the presence of jelly-precipitating factor had the same disturbing effect on the demonstration of the jelly-dissolving factor as was reported by Hultin *et al.* Hathaway *et al.* reported that they could spin down the jelly-precipitating activity. This corresponds to the results obtained by Runnström *et al.* (1955). They found the jelly-precipitating factor in the microsome fraction but also in the supernatant. A treatment of the microsome fraction with ribonuclease caused a further release of jelly-precipitating factor.

In summary it may be said that there is evidence of an enzymatic activity in the sperm that dissolves the jelly coat of the egg. The knowledge both of the character of this enzyme and the extent of the splitting is unsatisfactory. The splitting involves probably a certain not too deep-going depolymerization. The splitting enzyme may be present both in the spermatozoon and in the sperm liquid. Aketa (1961) demonstrated that the jelly coat does not play a role in the acid formation ensuing upon fertilization of the sea urchin egg. This is in keeping with the view that the breakdown of the jelly coat occurring under the impact of the spermatozoa does not lead to a pronounced splitting (scission) of the jelly coat substance. The question should, however, be reinvestigated with rather heavy sperm suspensions.

The "Je Ppt F(Sp)" is obviously a basic protein (T. Hultin, 1949); the same seems to hold for J Ppt F(E), as the results of Runnström and Monroy (1950) indicated. Hultin *et al.* (1952) regarded J Ppt F(E) as identical with the membrane-toughening factor of Motomura (1950), who gave the final demonstration of the basic character of this substance which he designated as "colleterin" (1957). This writer would prefer to maintain the descriptive designation, fertilization membrane-toughening factor, abbreviated, *e.g.,* Fmb tough F(E).

A change in the physical character of the jelly coat was also brought about by addition of certain substances, like heparin (Runnström and Wicklund, 1950; Harding, 1951) and the potent fertilization inhibitor from *Fucus,* Fe Inh (Fu) (see Harding, 1951; Runnström and Hagström, 1955; Esping, 1957a, 1957b; Branham and Metz, 1959; and Metz, 1961). The Fe Inh (Fu) counteracts the breakdown of the substance of the jelly coat by spermatozoa. Removal of the jelly coat reduces the inhibitory action of Fe Inh (Fu). The treatment of the naked egg with the inhibitor still brings about a considerable inhibitory effect (Runnström and Hagström, 1955). The same was the case with heparin (Harding, 1951). In view of the advantage of working with defined agents, chondroitin sulfate and germanin were also tested and were found to act as fertilization inhibitors in a manner very similar to that of Fe Inh (Fu) (Wicklund, 1954; Hagström *et al.,* 1957). Chondroitin sulfate impairs, for example, the dissolution of the jelly coat by spermatozoa.

This brief survey shows that the jelly coat may be affected by two different groups of substances, namely (1) by basic proteins, (2) by substances of acid character that are able to cross-link the fine structure of the jelly coat. These substances inhibit the dissolution of the jelly coat and delay the penetration of the spermatozoa. In the present paper it will be demonstrated that the jelly coat has different properties in different phases of development of the female genital cell. Furthermore an attempt will be made to explore the mechanism of these changes, both by evidence reported below and by evidence from pertinent literature. It will follow that lively interactions occur between so-called extraneous coats and the cytoplasm.

MATERIAL, METHODS

The egg and sperm of *Paracentrotus* (Pa.) *lividus, Arbacia* (A.) *lixula* and *Psammechinus* (Ps.) *microtuberculatus* and *Ps. miliaris* served as the main material in this research. The three first mentioned species were made available at the Stazione Zoologica, Naples, the last mentioned at the Kristineberg Zoological Station (Swedish west coast). The ovaries were cut open and the outflowing eggs were collected in sea water and thereafter passed through bolting silk. The eggs were further washed by suspending them in 300–500-ml. beakers. The sperm was removed from the testicles without dilution and then brought into small tubes that were stoppered and kept in a refrigerator. Two drops of the dry sperm were suspended in 5 ml. sea water and further diluted according to the type of experiment carried out (1:5; 1:10 etc.). An egg suspension was inseminated by rapid mixing with the diluted sperm. The jelly coat was removed from the eggs either by treatment with acid sea water (pH 5.2), following the direction of Vasseur (1948), or by filtration through gauze under pressure as described by

Markman (1958). This latter method was also used in order to estimate the mechanical resistance (consistency) of the jelly. In the experiments with basic stains or protamin sulfate the mixture of egg suspension and solution of the substances was made in a shallow concave dish but the observations were made after putting the oocytes or eggs between slide and coverslip. Between the observations, the slides were kept on stands enclosed in a container in which the atmosphere was kept moist. The stains used were brilliant cresyl blue (E. Merck) and toluidine blue (Gurr), the protamine sulfate was from Light and the reduced glutathione from Eastman & Co. A Leitz polarization microscope, furnished with a Köhler compensator, was used for the observations of the positive or negative sign of the birefringence with reference to the radial direction of the egg. The size of the figures (phase contrast) is *ca.* 340 × object.

The oocytes of the first order will be designated below as "oocytes" or "resting oocytes," whereas oocytes of the second order will be called "oocytes in maturation divisions" or "maturing oocytes."

For convenience the designation, "mature eggs," will be used for those which have completed the maturation division (meiosis); on the other hand the designations, "underripe," "ripe," etc. will refer to the changing state of cytoplasm that may be observed after meiosis.

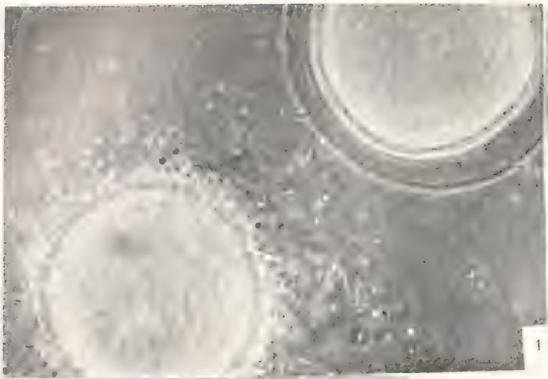
Results

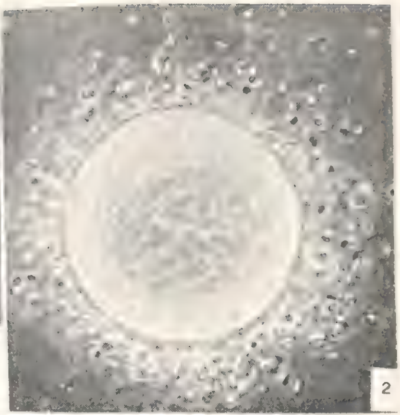
a. Effect of the sperm on the jelly coat of the egg

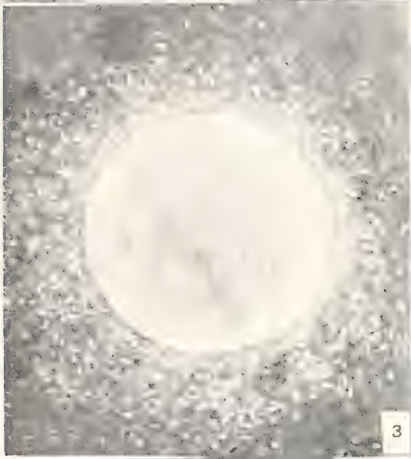
In one experiment, eggs of *Ps. microtuberculàtus* were washed repeatedly and then inseminated with a heavy sperm suspension, 5–6 × 10^6 per ml. The spermatozoa attacked the jelly coats of the mature eggs as in the experiments of Hartmann and Schartau (1939) on *Arbacia* eggs. In the present experiment, 70–80% of the eggs had lost their jelly coats 10–15 minutes after insemination. In the eggs retaining their jelly coats, very few spermatozoa were present. These were able to move around and in some instances actually left the jelly coat. After the treatment, the jelly coat offered a low resistance to the movements of the spermatozoa. The same phenomenon was readily observed also in the other species studied in this respect, *Ps. miliaris* and *Pa. lividus.*

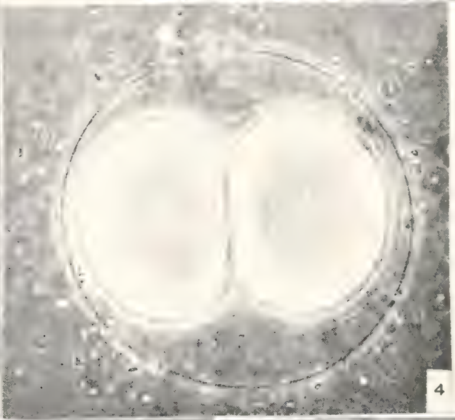
In contrast, the oocytes did not loose their jelly coats on insemination. Great numbers of spermatozoa were caught in the oocyte jelly coats, particularly in the inner zone. There were fewer spermatozoa in an outer zone (see Figure 1, representing an oocyte of *Pa. lividus,* about 30 minutes after insemination). The width of the jelly coat was about 50 μ (diameter of the oocyte is 94 μ). In the same figure a mature fertilized egg is represented. Very few spermatozoa were present in the jelly coat of the egg. Figure 2 gives another rather typical picture of an oocyte with accumulation of spermatozoa.

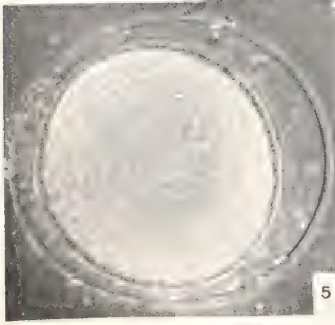
The spermatozoa invaded the jelly coat of the oocyte without difficulty. A number of them penetrated to the surface of the oocyte and there caused the formation of reception cones, the structure and behavior of which were recently examined by Runnström (1963); see that paper for references to previous literature. Upon heavy insemination, most of the spermatozoa were immobilized in the jelly coat. The heads of these spermatozoa often became spherical; the middle piece and tail remained unchanged so far as can be seen with the light microscope.

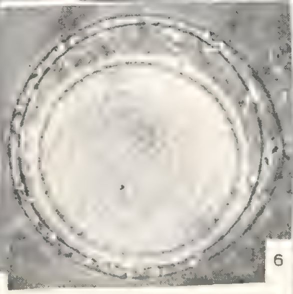

1


2


3


4


5


6

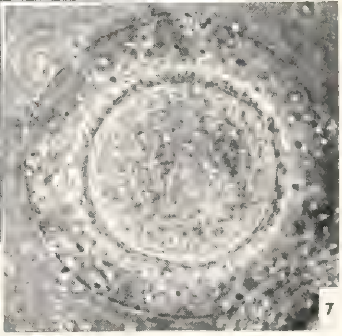

7

FIGURE 1. An oocyte and a ripe egg of *Paracentrotus lividus;* 30 minutes after insemination. 310 ×.

FIGURE 2. Oocyte of *Paracentrotus lividus, ca.* 25 minutes after insemination. 330 ×.

FIGURE 3. Oocyte of *Psammechinus microtuberculatus* in meiotic division. Penetration of several spermatozoa has provoked the formation of mitotic figures. 325 ×.

FIGURE 4. Egg of *Psammechinus microtuberculatus* about 70 minutes after insemination. 325 ×.

FIGURE 5. Egg of *Paracentrotus lividus,* four minutes after fertilization. 350 ×.

FIGURE 6. Eggs of *Paracentrotus lividus,* two minutes after insemination, exposed to 0.01% brilliant cresyl blue, reception cone visible in the proximal region of the egg. 310 ×.

FIGURE 7. Oocyte of *Paracentrotus lividus,* two minutes after insemination, exposed to 0.01% brilliant cresyl blue; photographed 35 minutes later. 310 ×.

Within the jelly of the oocyte, the spherical heads showed a dark outline in phase contrast. Such spermatozoa were present in the oocytes represented in Figures 1 and 2.

Since Selenka (1878), it has been known that the jelly coat of sea urchin oocytes is pierced by very thin filaments, the function of which seems to be the connection of the oocytes with the follicle cells; possibly they mediate uptake of substances from the follicle cells (see further Lindahl, 1941, and Runnström, 1944). The filaments are evidently withdrawn or broken down before the maturation divisions. The presence of the filaments does not exclude penetration of spermatozoa to the egg surface with subsequent formation of reception cones and immobilization of the spermatozoa. Despite the presence of filaments the situation was thus similar to that represented in Figure 1. The thin reception cones (*cf.* Runnström, 1963, Fig. 5) may also penetrate the whole width of the jelly coat. In view of these facts, the jelly coat of the oocytes seems to be rather highly organized. It may consist of a rather rigid jelly pierced by channels through which filaments, narrow reception cones and spermatozoa may pass. The rather high rigidity of the jelly coat of the oocytes was demonstrated by the method of Markman (1958). Non-fertilized eggs of *Paracentrotus* were filtered under light pressure through gauze, the meshes of which were smaller than the diameter of egg + jelly. The presence or absence of the jelly coats was tested by bringing a concentrate of non-fertilized eggs on a slide. The surfaces of the eggs without jelly coats were in close contact, whereas those with jelly coats were separated by an interspace representing the width of the jelly coats. In several such experiments 90–99% of the mature eggs had lost, whereas all the oocytes present had retained, their jelly coats. It was ascertained that the jelly coats of the mature eggs showed no strong swelling; the width of jelly coat was approximately equal to the radius of the oocyte or mature egg.

In one experiment (January, 1963) 237 non-inseminated female gametes of *Ps. microtuberculatus* were examined among a still greater number that had passed the gauze filter; out of the sample examined, 46 (19.5%) were oocytes with intact jelly coats, 189 were mature eggs, of which 156 (82%) had lost the jelly coat. The percentage of mature eggs, the jelly coats of which resisted filtration, shows that these eggs belonged to a batch of a moderate degree of cytoplasmic underripeness.

The greater resistance of the jelly coats of the oocytes was observed also when the jelly coats of egg suspensions were repeatedly washed with sea water under much agitation.

Oocytes in maturation divisions have not yet developed the complete mechanism that protects against polyspermy (Brachet, 1921; Runnström and Monné, 1945). In some experiments, counts of the number of reception cones were tried; it could be roughly estimated that the number of reception cones formed in maturing oocytes corresponded to about one-tenth of those found in resting oocytes. The relation between spermatozoa and jelly coat in maturing oocytes is similar to that described for the resting oocytes. There is a strong accumulation of immobilized spermatozoa in the jelly coat. Sometimes they are more numerous in an inner zone of the jelly coat, as in the case of resting oocytes; more often, however, they are rather uniformly distributed in the jelly coat of the maturing oocyte, as shown in Figure 3 (67 minutes after insemination). In this instance also, the sperm heads often became spherical. Figure 4 represents a mature egg about 70 minutes after fertilization. It belonged to the same egg batch as that of the maturing egg of Figure 3. The jelly coat of the egg of Figure 4 contained fewer spermatozoa than the resting or maturing oocytes; moreover, they retained a certain mobility and none of them had assumed a spherical form. Nevertheless, eggs of this category were considered not to have attained full cytoplasmic ripeness. As described earlier (see Runnström, 1949; B. Hagström, 1955) for *Ps. miliaris* from the Swedish west coast, a state of underripeness of the eggs was often observed during the breeding season, June–August. The characteristic of this state is a low rate of fertilization (B. Hagström, 1955) partly due to an accumulation and immobilization of the spermatozoa in the jelly coat. In extreme cases the fertilization was blocked in a varying number of eggs.

A relatively mild form of underripeness may be present for a great part of the breeding season at Kristineberg. In these cases the eggs have a low rate of fertilization the first hour after removal of the eggs from the ovaries but gradually the rate of fertilization increased and attained a maximum 3–4 hours after removal from the ovaries (B. Hagström, 1955).

The littoral so-called Z-form of *Ps. miliaris* (Lindahl and Runnström, 1929) is exposed to variations in salinity and temperature. These external factors probably influence the rate of cytoplasmic ripening in this form. The so-called S-form of *Ps. miliaris* (from a depth of 30–40 meters) is living under more constant external conditions. The state of underripeness is limited here to the beginning of the breeding season. It may be found that in the latter half of August the ovaries contain plenty of eggs having undergone nuclear maturation that are not fertilizeable by highly motile spermatozoa from S- or Z-form. In *Ech. cordatum* analogous conditions were found; (the latter half of July is the period of cytoplasmic underripeness with full ripeness following in the early half of August). These indications are based on experience from several years but there may of course be variations with respect to dates and duration of the periods of cytoplasmic maturity.

Phenomena comparable to those observed in Kristineberg were revealed in observations on *Pa. lividus* and *Ps. microtuberculatus* carried out at the Stazione Zoologica, Naples, in December, 1960 and 1962. During this period the filtering of the eggs under pressure deprived the eggs of their jelly coats only to a limited extent. Also, during the spring season it was observed that accumulation of spermatozoa in the jelly coat occurred; concurrently the resistance against filtration was high. It seems natural to consider the state of the jelly coat during the period of underripeness as directly continuous with the state found in the oocyte and in the stage of meiotic divisions. The duration of the period of cytoplasmic underripeness is dependent on exterior conditions. Lindahl and Runnström (1929) found reasons to believe that in *Ps. miliaris* the sexual maturity was dependent on the average temperature during a certain period rather than on peaks of temperature.

A moderate accumulation of spermatozoa may occur within the jelly coat but the fertilization membrane may nevertheless be elevated from the egg surface.

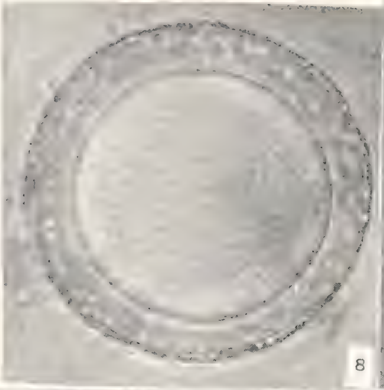
Such a case is represented in Figure 5 (*Pa. lividus*). Four minutes after insemination the fertilization membrane was, however, rather asymmetric; at the "proximal" pole (Runnström, 1962), the fertilization membrane was more elevated and more refractile than at the "distal" pole. Moreover, it was observed how the spermatozoa gradually left the jelly coat. There is an evident change in the property of the jelly coat starting from the moment when the proximal part of the fertilization membrane becomes smooth. As previously demonstrated (Runnström, 1962), this smoothing is dependent on the formation of the proximal concavity within which the penetrating spermatozoon is usually attached. The concavity formation is probably the expression of the secretion of a factor(s) that brings about the final incorporation of the cortical lamella (see Endo, 1961), and the final detachment of the fertilization membrane from other cortical elements and from the egg surface. At the distal pole, on the other hand, the fertilization membrane was less refractile and seemed granular, which signifies that the contact with the egg surface was not definitely broken (electron microscopic observations). In many cases the state represented in Figure 5 was only temporary and the smooth state of the fertilization membrane spread also to the distal side and simultaneously spermatozoa were released from the grip of the stiff jelly coat. These frequently repeated observations indicate that a secretion spreads from the proximal side of the egg in distal direction. This brings about the final detachment of the fertilization membrane from underlying components. The same secretion also changes the jelly coat so that its consistency decreases. The rate of smoothing of the distal region of the fertilization membrane varies in different batches of eggs. This may also be due to a certain state of the cytoplasm which depends in the last instance on exterior conditions. These may prolong or shorten a state of underripness. The fertilization membrane-toughening factor of Motomura (1957) is probably superimposed on the smoothing factor. The progress of jelly-toughening was followed with different methods by Motomura (1950) and by Markman (1958).

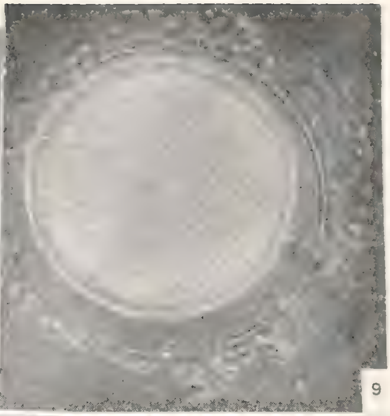
b. Precipitation of the jelly coat with basic stains

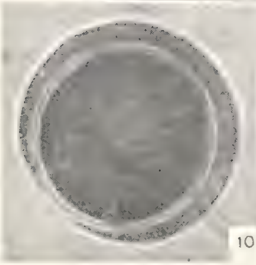
Oocytes and eggs of *Pa. lividus* were inseminated with a heavy sperm suspension. Two minutes later brilliant cresyl blue was added so as to make the suspension about 0.01% with respect to dye. There was an immediate precipitation of the jelly coat with a strong contraction (see Fig. 6). The spermatozoa that were present in the jelly coat were occluded and immobilized.

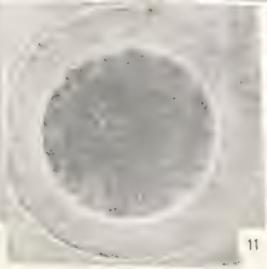
In some experiments the jelly coat shrunk down to a still thinner layer outside the fertilization membrane. In oocytes, a precipitation of the jelly coat was also manifested by a marked outline; on the other hand, there was no, or a very slight, contraction of the jelly coat, which remained many times thicker than the jelly coat in the fertilized egg. This is evident from a comparison of Figures 6 and 7. The latter represents an oocyte 37 minutes after insemination when the sperm-induced protrusions (see Runnström, 1963) have been retracted.

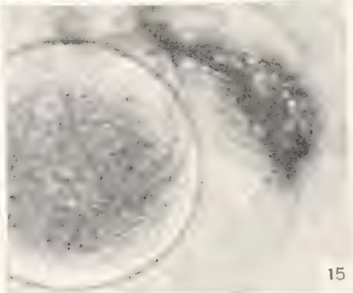
When toluidine blue dissolved in sea water was the jelly-precipitating agent the results were similar to those described. The concentration used was generally 0.01%. When the mature eggs were kept between slide and coverslip, they be-

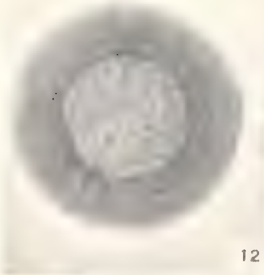

8

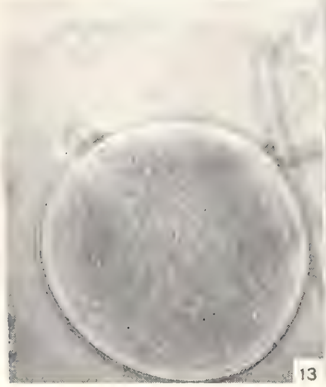

9


10


11


15


12


13

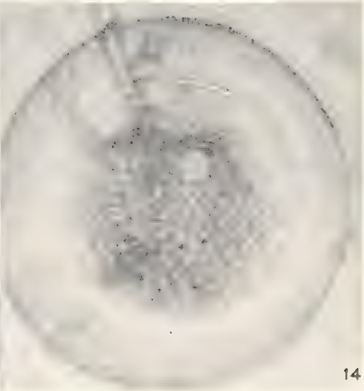

14

FIGURE 8. Egg two minutes after insemination, exposed to 0.01% toluidine blue in sea water, kept aerobically for 70 minutes. 380 ×.

FIGURE 9. Egg of *Paracentrotus lividus* two minutes after insemination, exposed to 0.01% toluidine blue, kept for 70 minutes under increasingly anaerobic conditions. 380 ×.

FIGURE 10. An unfertilized egg of *Arbacia lixula* brought into a 0.01% solution of brilliant cresyl blue in sea water; photographed *ca.* 25 minutes later. 310 ×.

FIGURE 11. A fertilized egg of *Arbacia*, two minutes after insemination brought into a 0.01% solution of brilliant cresyl blue in sea water; the egg belonged to the same batch as the unfertilized egg in Figure 10. 310 ×.

FIGURES 12, 13 and 14. Non-inseminated resting oocyte, oocyte in meiosis and mature egg of *Psammechinus miliaris*, immersed in 0.004% toluidine blue in sea water and kept for 15 minutes under aerobic conditions. 350 ×.

FIGURE 15. Unfertilized egg of *Psammechinus miliaris* with contracted fibrous jelly coat subsequent to addition of a 0.02% solution of brilliant cresyl blue from the side of the coverslip. 350 ×.

haved in two different ways (1) the precipitated state of the jelly coat continued; or (2) a swelling of the jelly coat occurred. It was easily stated that the first corresponded to a maintained oxidized state of the dye (see Fig. 8). This was found when the eggs were located near the edge of the drop of egg suspension enclosed between slide and coverslip. In eggs with a more central location in the drop, the dye was reduced and swelling of the jelly coat occurred as a consequence (see Fig. 9). Eggs with precipitated jelly coats were often aggregated; after reduction of the dye the eggs separated.

The precipitated jelly showed a birefringence that was positive in the radial direction. Monné (1943) previously demonstrated that after addition of acridine orange, the jelly coat has a similar birefringence. The sign of the birefringence that develops after staining is opposite to that of the fertilization membrane. In the egg represented in Figure 8 neither the membrane nor the jelly coat showed any birefringence. By the strong contraction of the jelly, this was amalgamated with the fertilization membrane to such an extent that the two opposite birefringences canceled each other out, the retardations being approximately equal. If, on the other hand, the contraction of the jelly was weaker, one could distinguish the positive birefringence of the jelly coat as contrasted to the negative birefringence of the fertilization membrane. The thin border of the jelly coat of the oocyte was birefringent; the character of the birefringence was the same as in the mature egg. When the jelly coat swelled under anaerobic conditions (see Fig. 9), the birefringences disappeared. The coverslips were removed in one experiment of the kind just described. Due to the contact with air, the dye was reoxidized and a reprecipitation of the jelly coat occurred. Under such circumstances the precipitate often took the form of threads appearing in the medium surrounding the egg.

After the addition of the dye, the precipitation of the jelly coat of mature unfertilized eggs was less pronounced than in the previously fertilized eggs. While in these latter the jelly coats shrank to a narrow rim in 2–3 minutes after addition of the stain, the shrinkage was slower and more gradual in the mature non-fertilized egg; they could, for example, decrease in width by 20–30% in 10–15 minutes. The difference could be due to the presence of spermatozoa within the jelly coat of the fertilized eggs. These may, for example, give off their jelly-precipitating factor. On the other hand, the shrinkage is to a large extent reversible after the reduction of the dye. Moreover, the spermatozoa occluded in the jelly coat of fertilized eggs are not damaged as far as can be judged from their appearance. Nevertheless it cannot be excluded that the jelly-precipitating factor from sperm plays a certain role, particularly after the rather heavy insemination in the experiment to which the eggs in Figures 8 and 9 belong.

The different behavior of fertilized and unfertilized eggs could be studied with advantage in eggs of *Arbacia lixula*, in which the fertilization membrane is only slightly elevated upon fertilization. Eggs from the same female of *Arbacia lixula* were divided into two portions. One (a) was directly transferred to 0.01% brilliant cresyl blue in sea water; the second (b) was inseminated with a very weak sperm suspension and transferred two minutes later to the stain solution. Figures 10 and 11 represent one unfertilized and one fertilized egg that were brought together on the same slide and photographed *ca.* 25 minutes after being transferred to the dye solution. Under aerobic conditions, the fertilized egg became as usual more heavily stained. It is of interest to note that this was true for the jelly coat as well. In the non-fertilized egg both the cytoplasm and the jelly coat were only faintly stained. The contraction of the jelly coat was weaker than in the fertilized egg. In many cases the contraction of the jelly of the fertilized egg was even stronger than shown in Figure 10, so that the jelly coat formed a thin rim outside the membrane. In this and in other experiments any effect of the jelly-precipitating factor from the sperm was insignificant.

In a similar experiment, the fertilized portion of eggs was transferred to the dye solution 23 minutes after insemination. The eggs, including the jelly coats, were measured with a screw micrometer, beginning 7 minutes after transfer to the stain solution. No essential changes in diameter took place during the measurements. The difference noted depended wholly on the different degree of retraction of the jelly coat. The difference between the fertilized and unfertilized eggs should in fact have been greater, because in the fertilized eggs the width of the perivitelline space is included in the measured diameter. In *Arbacia,* however, this space is so narrow that it can be disregarded.

The following averages (micrometer scale units, each equal to 0.25 μ) were found:

Unfertilized mature eggs	406.6 ± 9.3	$(n = 18)$
Fertilized eggs	349.4 ± 9.3	$(n = 16)$

The difference and its standard error is 57.2 ± 13.2. The probability that the two groups of eggs belong to the same category is thus <0.01.

The capacity of the jelly of the oocyte (of 1st and 2nd order) to take up stain seems to be lower than in the mature egg. Moreover the stain may become reduced sooner than in mature eggs. In contrast, the cytoplasm of the oocyte is more heavily stained than in subsequent stages. The capacity of the cytoplasm to take up dye is evidently very high, whereas the reduction is slow, owing to this high capacity of uptake. Evidently the jelly coat contains dehydrogenases or is in contact with reducing systems contained in the egg surface, the effect of which becomes weaker after maturation. Probably, the degree of staining of the jelly coat depends primarily on its capacity of binding the dye. This capacity increases after the maturation divisions and again after fertilization. In the fertilized eggs the occluded spermatozoa may play a role for the reduction of the dye.

Continuous observations were made on some female gametes of *Ps. miliaris* that were immersed in 0.004% toluidine blue in sea water.

After 10–14 minutes, no rim was visible in the jelly coat of the oocyte (Fig. 12). In a maturing oocyte (Fig. 13), a rim was present but the jelly showed very little shrinkage. Finally, in a mature egg the width of the jelly coat had undergone a shrinkage of about 30%; a marked rim had appeared (Fig. 14). The staining was strongest in the mature egg. In the latter there was a tendency to formation of fibers. This was particularly pronounced if the dye was added from the side of a suspension of eggs enclosed between slide and coverslip (see Fig. 15). The jelly coat contracted and its bulk accumulated on the side of higher dye concentration. A rather dense framework of fibers appeared. The single fibers were not uniform. In the fibers two components are distinguishable in phase contrast. The one is thinner and strictly fibrous and the other is granular. In more contracted fibers the granules (diameter *ca.* 0.2 μ) became more closely adjacent and broadened slightly in a transverse direction. The granules may possibly correspond to sites with higher concentration of acid groups, which thus become centers of contraction under the effect of the basic stain. When fertilized eggs were stained from one direction, the framework of fibers in the jelly coat was usually still denser than in the non-fertilized eggs. Conversely, oocytes treated in the manner described showed a framework in which the contraction is much less than in the mature eggs. The fibers are more granular and the meshes larger.

The stained unfertilized eggs were inseminated. Although many spermatozoa were unable to penetrate the jelly, the eggs were fertilized, but no membrane elevation took place. On immersion of the eggs in hypertonic medium (2 ml. sea water + 0.5 ml. 2.5 N NaCl), a radially striated hyaline layer, some microns wide, appeared, constituting a reliable indication that the eggs were fertilized. The fertilization membrane covered the hyaline layer, but no birefringence was demonstrated in the fertilization membrane. In the course of 20–30 minutes, however, a gradual delamination of the two membranes occurred. This was particularly obvious after immersion of the eggs in hypertonic medium. Simultaneously, the fertilization membrane acquired its birefringence. Under normal conditions this occurs as early as one minute after fertilization. The treatment of eggs with basic dyes thus causes a considerable delay of delamination.

In certain cases the previous staining interrupted the spreading of the activation impulse so that only the region encompassing the site of sperm entry was fertilized.

c. Effect of protamin on the jelly coat

It is well known (see Runnström, 1949, p. 294) that the jelly coat surrounding the sea urchin egg is precipitated under the influence of basic proteins, *e.g.*, protamins. Such experiments were repeated with protamin sulfate. Eggs of *Pa. lividus* were inseminated and, two minutes later, brought into a 0.005% solution of protamin sulfate in sea water (pH regulated to about 8.0). After 2–3 minutes the jelly of the mature fertilized eggs had contracted to a thin rim covering the fertilization membrane. Spermatozoa were captured in the contracting jelly.

The jelly coat of inseminated oocytes behaved rather differently upon addition of protamin. A precipitated outline was visible at the border of the jelly coat, but there was no, or only a slight, contraction of the jelly coats which, as usual, contained numerous spermatozoa. Whereas the contracted jelly coat of the fertilized eggs had a width of about 2 μ, the jelly coats of oocytes were about 20–25 μ wide. In some of the oocytes no precipitation of the exterior outline was found. The same striking difference appears here between oocytes and mature eggs as in the experiments with dyes. The jelly coat of the non-inseminated did not differ appreciatively from that of inseminated oocytes.

In further experiments a lower concentration of protamin sulfate was used, *viz.* 0.001–0.0005%. In these the contraction process was slower than at the higher concentration of protamin sulfate but the end result was similar. It was now evident that the jelly coat of unfertilized mature eggs did not contract to the same extent as that of fertilized eggs. On the other hand, the contraction was considerably stronger than in the non-inseminated oocytes. In one experiment (1) oocytes, (2) unfertilized mature and (3) fertilized eggs of *Pa. lividus* were exposed to 8 × 10^{-4}% protamin sulfate for 8–10 minutes. After this time the width of the jelly coat was, in (1) 46 μ, in (2) 20 μ; in (3) the jelly coat formed a narrow rim. Measurements were made on three eggs of (1) and (2), respectively. Qualitative observations on a great number of eggs confirmed the results of measurements. Even when the jelly coat of unfertilized eggs showed a visible precipitated outer rim, certain spermatozoa were usually able to penetrate to the egg surface and bring about fertilization of the mature egg, whereas the penetration of spermatozoa into the jelly coat of oocytes did not seem to be hampered.

Usually the fertilization membrane did not elevate from the egg surface with the exception of a region around the site of penetration of the spermatozoon. In this region delayed plate- or rod-like cortical lamellae were often present in the perivitelline space (Runnström, 1948; Endo, 1952). When glutathione was added in the concentration of 0.015% the elevation of the fertilization membrane was more pronounced, even in the presence of protamin sulfate. The addition of glutathione seems to increase the formation of plates and rods that are thinner and more elongated than in the parallel test containing protamin sulfate only. Exposure of unfertilized *Paracentrotus* eggs to protamin (0.005–0.012%) interferes rather strongly with the development of the eggs. The exposure lasted, *e.g.,* for 30 minutes; the eggs were then washed and fertilized in pure sea water. The blastocoel was narrower than in the control eggs and also the further development showed a considerable delay and inhibition, whereas the development was rather normal in eggs that were fertilized and, 5 minutes after fertilization, immersed in the protamin solution; the exposure here also lasted for 30 minutes. The eggs exposed to protamin before fertilization showed stronger damage in the vegetal, as compared with the animal, region in which a certain enlargement of the acron and the ciliary tuft was often observed. In some tests the eggs were exposed to a protamin solution that in addition contained glutathione (0.015%) during the exposure of unfertilized eggs. Subsequent to fertilization the development of these eggs became more normal than after pretreatment with protamin alone. In the experiments briefly mentioned it was irrelevant whether the eggs were surrounded by a jelly coat or if the jelly coat had been removed by a previous exposure to acid sea water.

Discussion

The main observation reported in the present paper is the different behavior of the jelly coat toward added spermatozoa in oocytes or maturing oocytes on one hand, and mature eggs on the other. The spermatozoa are not able to depolymerize the jelly coats in the first-mentioned stages, whereas this occurs in the mature eggs. However, there is a stage of underripeness prevailing after the meiotic division. This stage may last for a longer or shorter period, according to the species and external conditions. During the time of underripeness the spermatozoa accumulate in the jelly coats, which are evidently very resistant to the depolymerizing action of the spermatozoa. This state is gradually changed into the fully ripe one. This transition may be observed in individual batches of eggs or in eggs from different females during the progress of the breeding season.

It is very probable that the process of cytoplasmic ripening runs continuously until a certain maximum has been attained. Perlmann (1956) found that the rate of fertilization in *Pa. lividus* from Naples increases during the spring to attain a maximum in June. Before this period the removal of the jelly coat brought about an increase in rate of fertilization, but during the optimum period, the rate did not depend on the presence or absence of the jelly coat, *i.e.*, the resistance offered by the jelly coats had a minimum. In all these experiments care must be taken that the spermatozoa are in optimum conditions (see Vasseur *et al.*, 1950; Tyler, 1950, 1953).[1]

The observations made on eggs subjected to basic dyes or proteins were carried out in order to get further information about the changes responsible for the described difference in interaction between spermatozoa and jelly coat. The result was that in the resting oocytes and such undergoing meiotic divisions, some masking substance protects the jelly coat both against the basic substance and against the depolymerizing action of the spermatozoa. The jelly contains numerous acid groups, as shown by Runnström *et al.* (1942). Moreover, Vasseur (1948) found that the acid group has the character of sulfate, esterified with a polysaccharide moiety, constituting about 80% of the jelly (see further Tyler, 1949). It seems thus probable that a basic substance, most likely a protein, with basic groups, would be present in the jelly coats of the oocytes including the stage of meiosis. This substance would be able to mask the sulfated polysaccharides against the action of the spermatozoa. It would be expected that the sulfated polysaccharides combined with basic proteins would be masked against the action of externally added basic substances.

Another possibility emerges from the data concerning the action of certain added inhibitors, as, for example, the *Fucus* inhibitor, chondroitin sulfate, heparin, germanine etc. (Wicklund, 1954; Runnström, 1957a). The addition of these substances brings the mature eggs into a state similar to that of underripeness. The spermatozoa are accumulated in the jelly coat, the rate of fertilization is very delayed, the fertilization membrane is not, or only asymmetrically, elevated; the ensuing cleavage is irregular. This is not only due to an effect of the jelly layer; even in eggs without jelly coats irregularities in membrane elevation may remain (Runnström and Hagström, 1955); the rate of fertilization increases but does not always attain the value prevailing in the non-treated mature eggs (Runnström, 1957a).

---

[1] The papers by Collier (1959) and by Haino and Dan (1961) show that even in dilute solution the jelly coat substance induces an acrosomal reaction that is much in excess of the one found in normal sea water. Haino and Dan made the interesting observation that the action of the jelly coat substance is dependent on the age of the spermatozoa. Furthermore, early in the breeding season the reaction was weaker than at its optimum part. This is rather similar to what has been said above about the seasonal dependence of the jelly-dissolving action of the spermatozoa, although above the interaction was considered more from the point of view of the egg than from that of the spermatozoon. The data suggest, however, the working hypothesis that acrosomal reaction and activation of the jelly-depolymerizing enzyme are closely connected events. In an earlier paper Dan (1954) showed that agglutination and acrosomal reaction in spermatozoa should be considered as two separate responses of the spermatozoa to the jelly substance. Runnström and Hagström (1955) showed that the "*Fucus* inhibitor" (for references, see Metz, 1961) inhibits both the jelly-depolymerizing action of the spermatozoa and their complete separation after agglutination. This suggests that the separation is due to the action of the jelly-depolymerizing enzyme of the spermatozoa. When the spermatozoon has attached itself definitely to the cytoplasmic surface of the egg, the cortical changes start even if the further penetration of the spermatozoon is prevented (Runnström, 1957b).

The reaction of the spermatozoon with the jelly coat and that with the cytoplasmic surface of the egg are probably mediated by different sites in the acron region of the spermatozoon as may be inferred from Perlmann's work (1956, 1957, 1959) and from work of Baxandall, Perlmann and Afzelius now in press. The reactions in the surface may be of several kinds as follows also from a paper by Runnström and Kriszat (1960) that indicates the presence of a reception and an activating system in the egg surface.

The added inhibitors probably cross-link the micelles of the jelly coat. They may further inhibit the jelly-dissolving sperm-enzyme in competition with the jelly substance and in this way block or delay the penetration of the spermatozoa through the jelly coat. It is of interest that jelly substance added in relatively high concentration to the egg suspension also inhibits the sperm penetration (Runnström and Wicklund, 1950). As follows particularly from the work of Hagström (1956a, 1956b, 1956c) the jelly coat substance is usually a barrier for the spermatozoa. This barrier can only be broken down by certain of the spermatozoa. The jelly coat exerts thus a sieve-like, selecting action on the spermatozoa but, as follows also from Perlmann's observations referred to above, the selecting effect decreases with progress of the breeding season. Perlmann (1956, 1957, 1959) prepared antibodies against the jelly coat of *Paracentrotus*. The anti-jelly antibodies precipitated the jelly coat in a way very similar to the effect of basic protein. The antigens of the jelly coat are distinct from the egg antigens, although substances from the egg may enter the jelly coat, probably through a sort of secretion of diffusion. Perlmann's (1956) observations showed that the reaction of the jelly coat against anti-jelly serum varied but seemed to be extremely well correlated with the ripeness of the eggs. The oocytes only rarely showed any precipitation reaction. Perlmann explained the increased reactivity of the jelly coat to its specific antibodies by an unmasking of antigenic groups during maturation. Moreover, the reaction between jelly layer and anti-jelly serum is very low when the batch of eggs used in the experiment contained a relatively high percentage of oocytes. Even if the eggs have undergone their maturation divisions a batch of this character belongs to the underripe type. Perlmann infers from his results that the reaction between jelly and anti-jelly serum is causally related to an increased capacity of the jelly coat for letting homologous sperm pass. The maximum immune response was obtained during the optimum period in June referred to above.

It is obvious that there is a parallelism between reactivity of the jelly coat to anti-jelly serum and its accessibility to the jelly-dissolving sperm enzyme. Both sites of activity seem to be masked by the same substance or complex of substances. The parallelism can be extended to the basic substances, dyes and protamin sulfate. They do not precipitate the jelly coats of oocytes and maturing eggs but those of mature eggs. According to personal communication, Perlmann tested in 1956–1957 the effect of different sulfated polysaccharides, chondroitin sulfate, dextran sulfate, etc. on the reaction between the jelly and anti-jelly serum. No inhibition of the immune reaction was demonstrated. Conversely, the added sulfated compounds have a delaying effect on fertilization depending to a large extent on the increased resistance of the jelly coat. The spermatozoa do not constitute the limiting factor in experiments with sulfated polysaccharides. The motility and life span of the spermatozoa are considerably prolonged in the presence of the mentioned substances.

As already suggested, the addition of the sulfated compounds may increase the cross-linking within the jelly coat. This may increase the resistance to the sperm penetration that prevails even under normal conditions. Moreover, after the addition of the foreign substances the jelly-dissolving enzyme of the spermatozoa may meet a substrate beyond its range of specificity. Basic substances have not yet been studied with respect to eggs made refractory by added sulfated compounds.

More work is needed before definite conclusions can be drawn, but it is evident that, in oocytes, the reaction both between jelly and basic substances, and between jelly and anti-jelly serum are blocked. The simplest explanation is that the reacting sites are masked by basic proteins, secreted from the eggs. Maturation should then imply that the basic proteins in the jelly coat are gradually broken down. In fact, the maturation of the egg finally leads to a dissolution of the vitelline membrane which is an extremely sensitive indicator of proteolytic activity (see Runnström and Kriszat, 1960).

In fact, Lundblad (1950, 1954) found that proteolytic enzymes may be present in the jelly substance and they may even be released to the medium. Lundblad (1950) first believed that the proteolytic enzymes were components of the jelly coat but later arrived at the conclusion that the proteolytic enzymes leak out from the surface of the unfertilized egg. The enzyme mainly involved is E 2, an enzyme with its pH optimum about neutral reaction. This enzyme is inhibited by SH-compounds (Lundblad, 1954). It is activated upon fertilization but may possibly be deactivated by the release of a compound with free SH-groups. As long as E 2 dominates, the fertilization membrane is still connected with underlying structures. Moreover, the jelly coat remains dense and captures spermatozoa. The release of an SH-compound would, however, inhibit E 2, but bring about an activation of E 1 and E 3 that are present in the egg surface. In *Pa. lividus,* the changed situation begins about 30 seconds after insemination. It brings about the smoothing of the fertilization membrane, starting on the proximal side (Runnström, 1962). Moreover, a swelling of the jelly coat occurs so as to release the captured spermatozoa. This change follows closely the smoothing of the fertilization membrane (see Fig. 5). Oxidation of the SH-groups in the egg surface with the high potential dye, porphyrindin, prevents the swelling of the jelly coat and the elevation of the fertilization membrane. The spermatozoa remain captured (Runnström, 1957b). Conversely, treatment of eggs with glutathione increases the elevation of the fertilization membrane and the swelling of the jelly. It seems thus probable that several substances are exuded from the egg upon fertilization and even invade the jelly coat.

Immers (1961c) showed in eggs of *Echinus esculentus* that sulfated mucopolysaccharides escape from the egg surface concurrently with the formation of a wider perivitelline space between fertilization membrane and egg surface. Ishihara (personal communication) has elaborated these observations for other species; moreover, he demonstrated that the acid polysaccharides are chemically of a type different from those present in the jelly coat (see also Immers, 1952). Probably there may, besides the acid polysaccharides, also be other substances exuded, of which some may be held back by the fertilization membrane. Others may penetrate this latter and are thus able to act on the jelly coat.

The acid formation accompanying the release of the acid polysaccharides (Runnström and Immers, 1956; Aketa, 1963; Ishihara, personal communication) may also serve as an activator of enzymes like cathepsin II (Lundblad, 1954) that have a rather low pH-optimum.

Borei (1948) found, in work with *Ps. miliaris* from the vicinity of Kristineberg, that a relatively high rate of respiration prevailed in the unfertilized eggs immediately after their removal from the ovary. When the eggs remained in contact with sea water, the rate of respiration gradually decreased until the attainment of an asymptotic value. The post-fertilization rate of respiration was always the same irrespective of the rate prevailing before fertilization. Yasumasu and Nakano (1963), Ohnishi and Sugiyama (1963) found, however, a constant low rate of respiration in the unfertilized sea urchin egg, even when the interval between removal of the eggs from the ovaries and the fertilization was brief.

As Ohnishi and Sugiyama point out, Borei's results have thus not general validity. They are certainly to be explained by the mild type of underripeness mentioned in section 3 a. B. Hagström (1955) found that the eggs usually attain their maximum rate of fertilization 3–4 hours after removal from the ovary. In good agreement with this Borei found that the respiratory curve became almost horizontal four hours after removal of the eggs from the ovary. According to T. Hultin (personal communication) the incorporation of labeled amino acids was usually higher in unfertilized eggs of *Ps. miliaris* (Kristineberg) than in unfertilized eggs from the high season of *Pa. lividus* (Naples). In underripe eggs, low rate of fertilization seems to be combined with rather high rate of respiration and of incorporation of labeled isotopes. In certain species and under optimal external conditions the transition state may be very short.

The controversy may partly depend on variations in the rate of decline of respiration from the high level found in oocytes (Lindahl and Holter, 1941) and the low level prevailing in mature unfertilized eggs. In different species and under different conditions, the decline may be steep or more gradual.

Possibly induction of spawning by means of KCl-injection is more selective with respect to ripe eggs than the removal of the ovaries into sea water, the method used by the Swedish workers. Hultin and Hagström (1956) found that the eggs of one female of *Ps. miliaris* (Kristineberg) sometimes formed several statistical groups probably representing different stages of ripeness.

The spermatozoa enter without difficulty into the jelly coat of the oocyte that has been exposed to protamin sulfate. This suggests that the jelly coat may be perforated by radially directed pores. As recalled above thin filopodia penetrate the jelly coat in the oocyte of first order. The filopodia are withdrawn prior to the meiotic divisions. On the other hand, filopodia and reception cones appearing subsequent to insemination were sometimes seen to coexist. There may be a certain reorganization of the fine structure of the jelly coat following the meiotic divisions. The gradual maturation of the jelly coat and cytoplasm represents steps of this reorganization. After the meiotic divisions the precipitation of the jelly coat with basic substances delays considerably, or may even completely bar the penetration of spermatozoa. At the same time there is an increased tendency to formation of fibers that have a tangential direction. The changes referred to might indicate a rearrangement of micelles within the jelly coat from a more radial to a more tangential orientation.

Under anaerobic conditions the dyes are reduced within the jelly coat. The capacity of reduction of the jelly coat seems to be greater in oocytes than in mature eggs. The present observations give, however, no clue to the nature of the reducing systems. It is of interest that the degree of swelling of the jelly coat is dependent on the oxidation-reduction state of the dye, a situation that may be found also within the cytoplasm (diphosphopyridine nucleotide being a base in oxidized but not in reduced state).

Some incidental results obtained may also be briefly commented on. Treatment of unfertilized eggs with basic proteins was more injurious than a similar treatment initiated only some minutes after fertilization. The explanation is probably that, before fertilization, acid polysaccharides are bound to the egg surface and that they are, to a large extent, released from the egg surface upon fertilization (see Immers, 1961c). It is conceivable that the basic substances interfere more seriously in the former case. After fertilization the main part of the acid polysaccharides is found in extraneous structures, the fertilization membrane, the hyaline layer and the perivitelline space. Even if these structures react to fuller extent with the basic proteins, their effect on the development is small. This may be due to the continuous secretion process occurring in the surface of the embryos (Immers, 1961c). The tendency to animalization apparent in eggs treated with basic protein before fertilization indicates that the vegetal region has a differential susceptibility to the effect of the precipitation of acid polysaccharides. This may be in keeping with the view expressed by Motomura (1960) and Immers (1961a, 1961b) regarding the importance of mucopolysaccharides in the primary invagination of the archenteron.

The results of the present research show how dependent the properties of extraneous coats may be of the phase of development of the female gametes. The properties of the coats, on the other hand, may be of great importance for the relations of a cell to the surrounding medium, including other cells. The brief remarks in the preceding paragraph point to the importance of the properties of the cell coats in morphogenesis (for further references, see Gustafson and Wolpert, 1963).

The experimental part of this work has been carried out at Stazione Zoologica, Naples, and at the Kristineberg Zoological Station (Sweden). The writer is greatly indebted to Dr. Peter Dohrn, Naples, and to Dr. B. Swedmark, Kristineberg, for their generous help and support. Furthermore, the writer expresses his deep gratitude for the financial help received from the Swedish Natural Sciences Research Council and the Swedish Cancer Society.

SUMMARY

1. The spermatozoa are able to depolymerize the jelly coat of mature eggs of sea urchins (*Psammechinus microtuberculatus*, *Ps. miliaris*, *Paracentrotus lividus*, *Arbacia lixula*), whereas that of resting oocytes or oocytes in meiosis is resistant. As follows from a survey of previous work (see Introduction) the depolymerizing

action of the spermatozoa is probably of enzymatic nature. The mechanical resistance of the jelly coat was studied by filtration of the female gametes through gauze of appropriate mesh width. The jelly coats of the oocytes proved to be more resistant than those of mature eggs.

2. The state of the jelly coat does not change abruptly at the conclusion of the meiotic divisions but a transition state prevails for a varying length of time. This is the state of cytoplasmic underripeness, the duration of which depends on exterior conditions and probably also on the species. The ripe state involves a maximum rate of fertilization and—as may be inferred from Borei's work (1948) a minimum rate of respiration.

3. Basic dyes (toluidine blue and brilliant cresyl blue) and protamin sulfate cause a strong precipitation and contraction of the jelly coat of fertilized egg. The effect is less pronounced in unfertilized mature eggs and very weak in resting oocytes and oocytes in meiosis. It is inferred that in the oocytes there is a masking of the acid groups of the jelly coat. The masking declines only gradually according to the rate of cytoplasmic ripening that is connected with a release of certain substances from the egg cytoplasm. These influence the jelly coat. Particularly a change of the jelly coat occurs concurrently with the smoothing of the fertilization membrane. There is a parallelism between the masked state in the oocytes described above and their lack of reactivity with anti-jelly serum that was found by Perlmann (1956, 1957, 1959). He stated that the precipitating effect of the anti-jelly serum had a maximum when the eggs were in their highest state of ripeness (maximum rate of fertilization).

4. If basic dyes precipitating the jelly coats are reduced, a swelling of the jelly coats occurs. Eggs subjected to basic proteins before fertilization have a less normal development than eggs treated by the same dose of basic proteins after fertilization.

LITERATURE CITED

Aketa, K., 1961. Studies in the production of the fertilization acid in sea urchin egg. I. Acid production at fertilization and activation, and the effect of some metabolic inhibitors. *Embryologia*, **5**: 397–405.

Allen, R. D., and B. E. Hagström, 1955. Interruption of the cortical reaction by heat. *Exp. Cell Res.*, **9**: 157–167.

Borei, H., 1948. Respiration of oocytes, unfertilized eggs and fertilized eggs from *Psammechinus* and *Asterias*. *Biol. Bull.*, **95**: 124–150.

Brachet, A., 1962. Sur la fécondation prématurée de l'oeuf d'oursin. *Arch. de Biol.*, **32**: 205–248.

Branham, J. M., and C. B. Metz, 1962. Inhibition of fertilizin agglutination and fertilization in *Arbacia* by *Fucus* extracts. *Biol. Bull.*, **122**: 194–207.

Collier, J. R., 1959. The effect of homologous fertilizin on the sperm of *Strongylocentrotus purpuratus*. *Acta Embryol. et Morph. Exper.*, **2**: 163–170.

Dan, J. C., 1954. Studies on the acrosome. III. Effect of calcium deficiency. *Biol. Bull.*, **107**: 335–349.

Endo, Y., 1952. The role of the cortical granules in the formation of the fertilization membrane in eggs from Japanese sea urchins. I. *Exp. Cell Res.*, **3**: 406–418.

Endo, Y., 1961. Changes in the cortical layer of sea urchin eggs at fertilization as studied with the electron microscope. *Exp. Cell Res.*, **25**: 383–404.

Esping, U., 1957a. A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*. I. The preparation of the factor inhibiting fertilization. *Arkiv Kemi*, **11**: 107–115.

Esping, U., 1957b. A factor inhibiting fertilization of sea urchin eggs from extracts of the alga *Fucus vesiculosus*. II. The effect of the factor inhibiting fertilization on some enzymes. *Arkiv Kemi,* **11**: 117–127.

Gustafson, T., and L. Wolpert, 1963. The cellular basis of morphogenesis and sea urchin development. *Int. Rev. Cytol.,* **15**: 139–214.

Hagström, Britt, 1955. Studies in the maturation of underripe sea urchin eggs. *Exp. Cell Res.,* **9**: 313–318.

Hagström, B. E., 1956a. Studies on the fertilization of jelly-free sea urchin eggs. *Exp. Cell Res.,* **10**: 24–28.

Hagström, B. E., 1956b. The effect of removal of the jelly coat on fertilization in sea urchins. *Exp. Cell Res.,* **10**: 740–743.

Hagström, B. E., 1956c. The influence of the jelly coat *in situ* and in solution on cross fertilization in sea urchins. *Exp. Cell Res.,* **11**: 306–316.

Haino, K., and J. C. Dan, 1961. Some quantitative aspects of the acrosomal reaction to jelly substance in the sea urchin. *Embryologia,* **5**: 376–383.

Harding, C. V., 1951. The action of certain polysaccharides on fertilization in the sea urchin eggs. *Exp. Cell Res.,* **2**: 403–415.

Hartmann, M., 1956. Die Sexualität. Stuttgart.

Hartmann, M., and O. Schartau, 1939. Untersuchungen über die Befruchtungsstoffe der Seeigel. *Biol. Zentralbl.,* **59**: 571–587.

Hathaway, R. R., and L. Warren, 1961. Further investigations of egg jelly dispersal by *Arbacia* sperm extract. *Biol. Bull.,* **121**: 416–417.

Hathaway, R. R., L. Warren and J. Flaks, 1960. Egg jelly dispersal by *Arbacia* sperm extracts. *Biol. Bull.,* **119**: 319.

Hultin, E., and G. Lundblad, 1952. Degradation of starch, hydroxiethyl cellulose ether and chitosan by enzymes in spermatozoa and sperm fluid from *Psammechinus* and *Modiola*. *Exp. Cell Res.,* **3**: 427–432.

Hultin, E., and B. E. Hagström, 1956. The variability in the fertilization rate. *Exp. Cell Res.,* **10**: 294–308.

Hultin, E., G. Kriszat, S. Lindvall, G. Lundblad, J. Runnström, H. Lööw, E. Vasseur and E. Wicklund, 1952. On the interaction between the gametes of the sea urchin at fertilization. *Arkiv Kemi,* **5**: 83–89.

Hultin, T., 1949. Agglutination of sea urchin eggs by nucleoproteins. *Arkiv Kemi,* **1**: 419–423.

Immers, J., 1952. Carbohydrate components in unfertilized sea urchin eggs. *Arkiv Zoologi,* (2), **3**: 367–371.

Immers, J., 1961a. Comparative study of the localization of incorporated ^{14}C-labeled amino acids and $^{35}SO_4$ in the sea urchin ovary, egg and embryo. *Exp. Cell Res.,* **24**: 356–378.

Immers, J., 1961b. Incorporation of $^{35}SO_4$ in the sea urchin egg and larvae. *Arkiv Zoologi,* (2), **13**: 561–564.

Immers, J., 1961c. The occurrence of sulfated mucopolysaccharides in the perivitelline liquid of *Echinus esculentus*. *Arkiv Zoologi,* (2), **13**: 299–306.

Lindahl, P. E., 1941. Über experimentell hervorgerufene Riesenrichtungskörperchen sowie Bemerkung über das Ovarialei des Seeigels *Strongylocentrotus droebachiensis*. *Acta Zoologica,* **22**: 101–121.

Lindahl, P. E., and J. Runnström, 1929. Variation und Ökologie von *Psammechinus miliaris*. *Acta Zoologica,* **10**: 101–184.

Lindahl, P. E., and H. Holter, 1941. Über die Atmung der Ovocyten erster Ordnung von *Paracentrotus lividus* und ihre Veränderungen während der Reifung. *C. R. Trav. Lab. Carlsberg, Ser. Chim.,* **24**: 49–57.

Lundblad, G., 1950. Proteolytic activity in sea urchin gametes. I. Activity in untreated extracts. *Exp. Cell Res.,* **1**: 264–271.

Lundblad, G., 1954. Proteolytic activity in sea urchin gametes. IV. Further investigations of the proteolytic enzymes of the egg. *Arkiv Kemi,* **7**: 127–157.

Lundblad, G., and A. Monroy, 1950. Mucopolysaccharase activity of sea urchin sperm. *Arkiv Kemi,* **2**: 343–347.

Markman, B., 1958. Studies on the formation of the fertilization membrane in sea urchins. *Acta Zoologica,* **39**: 103–115.

Metz, C. B., 1961. Use of inhibiting agents in studies on fertilization mechanisms. *Int. Rev. Cytol.*, **11**: 219–253.

Monné, L., 1943. The induced birefringence of the jelly coat of the sea urchin eggs. *Arkiv Zoologi*, **35B**: nr. 3, 1–6.

Monné, L., and S. Harde, 1951. On the cortical granules of the sea urchin egg. *Arkiv Zoologi*, (2), **1**: 487–498.

Monroy, A., and H. Ruffo, 1947. Hyaluronidase in sea urchin sperm. *Nature*, **159**: 603–604.

Motomura, I., 1950. On a new factor for the toughening of the fertilization membrane of sea urchins. *Sci. Rep. Tohoku Univ.*, (4), **18**: 561–570.

Motomura, I., 1957. On the nature and localization of the third factor for the toughening of the fertilization membrane of the sea urchin egg. *Sci. Rep. Tohoku Univ. 4th Ser.*, **23**: 167–181.

Motomura, I., 1960. Secretion of mucosubstance in the gastrula of the sea urchin embryo. *Bull. Marine Biol., St. Asamushi*, **10**: 165–169.

Ohnishi, T., and M. Sugiyama, 1963. Polarographic studies of oxygen uptake of sea urchin eggs. *Embryologia*, **8**: 79–88.

Perlmann, P., 1956. Responses of unfertilized sea urchin eggs to antiserum. *Exp. Cell Res.*, **10**: 324–353.

Perlmann, P., 1957. Analysis of the surface of the sea urchin egg, by means of antibodies. *Exp. Cell Res.*, **13**: 365–390.

Perlmann, P., 1959. Immunochemical analysis of the surface of the sea urchin egg—an approach to the study of fertilization. *Experientia*, **15**: 41–52.

Runnström, J., 1944. Notes on the formation of the fertilization membrane and some other features of the early development of the *Asterias* egg. *Acta Zoologica*, **25**: 1–9.

Runnström, J., 1948. On the action of trypsin and chymotrypsin on the unfertilized sea urchin egg. *Arkiv. Zoologi*, **40A**, nr. 18: 1–26.

Runnström, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymology*, **9**: 241–326.

Runnström, J., 1957a. Surface layers of the sea urchin egg and their role in fertilization. *Festschr. Arthur Stoll, Sandoz A.G. Basel*: 850–868.

Runnström, J., 1957b. On the effect of porphyrexid and porphyrindin on the fertilization of the sea urchin egg. *Exp. Cell Res.*, **12**: 374–394.

Runnström, J., 1962. Effect of exposure to ribonuclease on the cortical changes occurring upon fertilization. *Exp. Cell Res.*, **27**: 485–526.

Runnström, J., 1963. Sperm-induced protrusions in sea urchin oocytes. A study of phase separation and mixing in living cytoplasm. *Devel. Biology*, **7**: 38–50.

Runnström, J., and L. Monné, 1945. On some properties of the surface layers of immature and mature sea urchin egg, especially the changes accompanying nuclear and cytoplasmic maturation. *Arkiv Zoologi*, **36A**: nr. 18, 1–26.

Runnström, J., and A. Monroy, 1950. The effect of egg extract on the surface of sea urchin eggs and spermatozoa. *Arkiv Kemi*, **2**: 405–416.

Runnström, J., and E. Wicklund, 1950. Formation mechanism of the fertilization membrane in the sea urchin egg. Inhibitory effect of heparin and jelly substance on clotting of the vitelline membrane. *Arkiv Zoologi*, (2), **1**: 179–194.

Runnström, J., and B. E. Hagström, 1955. Studies on the action of the "*Fucus* fertilization inhibitor" on sea urchin egg and sperm. *Exp. Cell Res.*, **8**: 235–239.

Runnström, J., and J. Immers, 1956. The role of mucopolysaccharides in the fertilization of the sea urchin egg. *Exp. Cell Res.*, **10**: 354–363.

Runnström, J., and G. Kriszat, 1960. The sperm reception and the activating system in the surface. *Arkiv Zoologi*, **13**: 95–112.

Runnström, J., A. Tiselius and E. Vasseur, 1942. Zur Kenntnis der Gamonwirkungen bei *Psammechinus miliaris* und *Echinocardium cordatum*. *Arkiv Kemi, Min. och Geol.*, **15A**: nr. 16, 1–18.

Runnström, J., B. E. Hagström and H. Löw, 1955. On the effect of ribonuclease on a jelly precipitating factor from the egg of the sea urchin, *Psammechinus miliaris*. *Exp. Cell Res.*, **8**: 235–239.

Selenka, E., 1878. Befruchtung des Eies von *Toxopneustes variegatus*. Leipzig.

Tyler, A., 1949. Properties of fertilizin and related substances of eggs and sperm of marine animals. *Amer. Naturalist*, **83**: 195–219.

Tyler, A., 1950. Extension of the functional life span of spermatozoa by amino acids and peptides. *Biol. Bull.*, **99**: 324.

Tyler, A., 1953. Prolongation of life span of sea-urchin spermatozoa, and improvement of the fertilization reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, Versene, DEDTC, oxine, cupron). *Biol. Bull.*, **104**: 224–239.

Vasseur, E., 1948. Chemical studies on the jelly coat of the sea urchin egg. *Acta Chem. Scand.*, **2**: 900–913.

Vasseur, E., 1951. Demonstration of a jelly-splitting enzyme at the surface of the sea urchin spermatozoon. *Exp. Cell Res.*, **2**: 144–146.

Vasseur, E., E. Wicklund and J. Runnström, 1950. Respiration and fertilizing capacity of sea urchin sperm in the presence of serum albumin and jelly coat solution. *Biol. Bull.*, **99**: 324.

Wicklund, E., 1954. The influence of some inhibiting substances on fertilization in the sea urchin egg. *Arkiv Zoologi*, **6**: 485–502.

Yasumasu, I., and E. Nakano, 1963. Respiratory level of sea urchin eggs before and after fertilization. *Biol. Bull.*, **125**: 182–187.

SUN COMPASS ORIENTATION OF PIGEONS UPON DISPLACEMENT NORTH OF THE ARCTIC CIRCLE

KLAUS SCHMIDT-KOENIG[1]

Dept. of Zoology, Duke University, Durham, N. C., and Max-Planck-Institut f. Verhaltensphysiologie, Abt. Mittelstaedt, Seewiesen, Germany

The use of the sun compass by animals poses interesting and thus far unsolved problems when we consider animals which migrate over many degrees of latitude. Drastic changes in the sun's rate of change of azimuth, direction of movement, etc., are encountered as one changes latitude. The general aspects of this problem have been discussed in more detail by Braemer (1960) and in a recent paper in this journal by Schmidt-Koenig (1963).

Birds in particular are known to travel over long distances. Examination of the response of directionally trained birds upon long distance latitudinal displacement may help to answer the relevant questions.

Two experimental series of experiments on sun compass orientation in birds upon large latitudinal displacement have so far been reported. Hoffmann (1959) displaced starlings (*Sturnus vulgaris*) from Wilhelmshaven, Germany, to Abisco, Sweden. Schmidt-Koenig (1963) took homing pigeons from Durham, N. C., to Belem, Brazil, and to Montevideo, Uruguay. The results of another experiment involving large northward translocation of homing pigeons from Durham, N. C., to Barrow, Alaska, will be dealt with in this paper.

The work reported in this paper was supported by a grant from the National Science Foundation (No. G 19849 to P. H. Klopfer and K. Schmidt-Koenig) and by a contract from the Office of Naval Research (No. 301–618 with Duke University). I am much indebted to Mr. Max Brewer, Director, Arctic Research Laboratory, Barrow, Alaska, and his staff for their extremely helpful cooperation and assistance. I want to thank Peter H. Klopfer for his steady helpfulness and support, and for reading and improving the manuscript.

METHODS AND ANIMALS

A description of the semi-automatically operating apparatus for training and testing, the mathematical procedure and the calculation of solar data was given previously (Schmidt-Koenig, 1963). Upon return from the South America experiment (op. cit.) the southward training of pigeons No. 1, 3, 6, and 7 was reinforced at Durham, N. C., in the spring of 1962. In addition, pigeons No. 10 and 14, also offspring of the Wilhelmshaven strain bred at Duke University, were newly trained to the east. Pigeons No. 3, 6, and 14 were shipped to Montevideo for another series of transequatorial tests. Unfortunately, Uruguayan officials sacrificed the birds upon arrival. Pigeons No. 1, 7, and 10 remained for the Alaska experiment.

---

[1] Present address: I. Zoologisches Institut der Universität, Göttingen, Germany.

Experiments and Results

The translocation experiment to Alaska was planned to fall around the summer solstice of 1962. On June 7, 1962, the birds were trained for the last time at Durham (36° 00′ N; 78° 56′ W). The travel to Barrow, Alaska (71° 10′ N; 150° 41′ W), involved a longitudinal time shift of about 5 hours counterclockwise. In order to facilitate the adjustment, experimenter and birds traveled by rail from Durham, N. C., to Seattle, Washington, from June 9, to June 13, 1962. The travel from Seattle to Barrow took another two days of waiting and flying. In Barrow, the birds were housed in their transportation crate in a greenhouse of the Arctic Research Laboratory. They were covered from four hours before midnight to four hours after midnight until June 18, 1962. From then on they were exposed to the permanent Arctic day. The location of the crate in the greenhouse prevented the birds from directly seeing the sun from about two hours before to three hours after midnight.

A site without tall landmarks around it was found just west of the Arctic Research Laboratory. The minimum altitude of the midnight sun at Barrow was 5°. For the tests around midnight, the aluminum palisade of the experimental apparatus was replaced by a double-layer gauze screen. Tests were performed from June 17 through June 29, 1962, with one or two, exceptionally three, sessions per 24 hours. There was no training. The weather was unusually favorable. Only on a few days was no sun shining at all.

It was intended to obtain a score from each bird for every half hour of the 24 hours of the day, but, when the dichotomy of behavior at "night" appeared, more attention was paid to the critical hours around and after 6 P.M. The performance of the birds (No. 10 recalculated as if trained to the south) and the sun azimuth curve for the summer solstice for the experimental and the home location are given in Figure 1. During "day" time the birds allowed by and large for a northern hemisphere sun azimuth. The present method is, however, too insensitive and the total number of scores is too small to tell whether the birds referred to the Barrow or the Durham azimuth. But the approach is sufficiently sensitive to reveal a clear dichotomy of behavior at "night." The birds referred to the sun as if it were moving clockwise (*i.e.,* through north) as well as if it were moving counterclockwise (*i.e.,* through south) at "night" time. The former is demonstrated by the extension of scores along the Barrow sun azimuth curve, the latter by the scores branching off to the upper right in Figure 1.

Discussion

Previously, certain taxa of animals have been found to allow either for a sun moving clockwise at night ("bee pattern") or for a sun moving counterclockwise at night ("*Talitrus* pattern"). Examples of the former are bees (Lindauer, 1957), certain riparian spiders (Papi and Syriämäki, 1963), starlings (Hoffman, 1959),

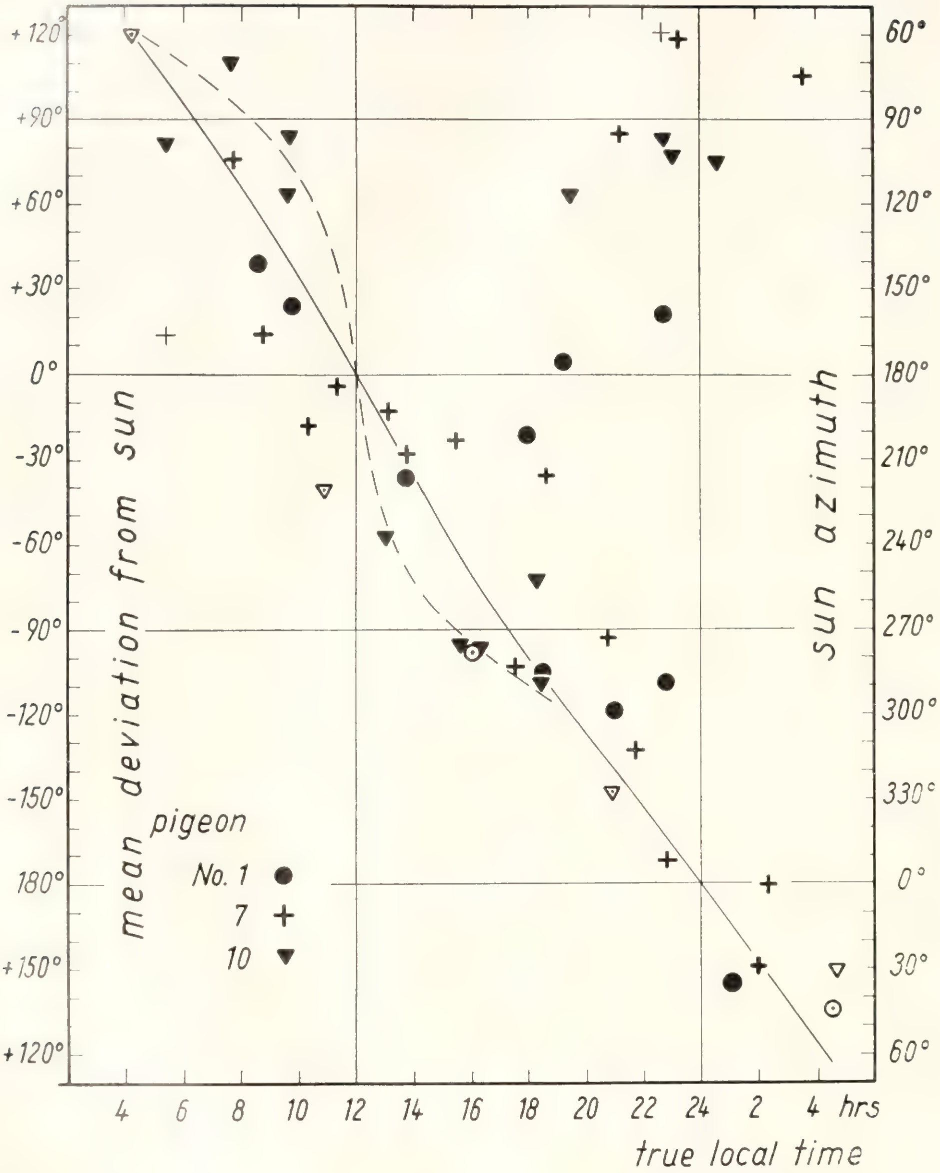


FIGURE 1. The performance of the three pigeons (key inserted at the lower left) at Barrow, Alaska. Each score represents the direction of the mean vector of all unrewarded choices during one examination session performed at the time of day (true local time = TLT) indicated on the abscissa and plotted as angle to the actual sun azimuth position (left coordinate). Black symbols represent 6–20 choices non-random at $p \leq 0.05$ (Rayleigh test), light symbols those random at $p > 0.05$. Open symbols with a central point summarize less than 6 choices. The solid diagonal line gives the sun azimuth at Barrow on June 21, 1962, the dotted line that at Durham, N. C., on the same date. The right coordinate designates true sun azimuth positions.

and fish (Braemer, 1959; 1960). Examples of the latter are a number of arthropods (Pardi, Papi, Birukow and others; see Pardi, 1960, and Birukow, 1960, for references), and pigeons (Schmidt-Koenig, 1961).[2] Papi and Syriämäki (1963) found two different patterns in different populations of one species of spiders (*Arctosa cinerea*). Finnish populations exhibited the bee pattern, Italian populations showed no definite orientation at night but some tendency for the *Talitrus* pattern. The results given in the present paper demonstrate that individual pigeons may exhibit both the bee pattern and the *Talitrus* pattern.

It is obvious to ask which factors trigger the alternative behavior. At least the data on birds may suggest this to be a function of the day-night conditions the birds are living in. The two pigeons of Schmidt-Koenig (1961) lived in a 12/12 hr. LD cycle (that was shifted to permit tests under the natural sun). Hoffmann's (1959) starlings, which allowed exclusively for a clockwise movement of the sun, were living in the permanent Arctic day for more than five weeks (that they were retrained in the testing area may also be relevant). The pigeons showing both patterns (Fig. 1) lived in the permanent Arctic day at Barrow for only 10 days. This type of behavior may, therefore, perhaps be interpreted as a transition from one pattern to the other. The bee pattern is certainly the only useful pattern. On the other hand, starlings have not yet been tested at "night" when living in an LD cycle and it is not known whether the *Talitrus* pattern can be produced at all.

The two-component theory of the sun compass (Mittelstaedt, 1963) is capable of explaining the alternative behavior even in individuals. The theory predicts the branching-off of the alternative behavior for 18:00 hours. The data from the pigeons (in this paper and in Schmidt-Koenig, 1961) do not seem to be fully consistent with this prediction. The entire branch of the (nocturnal) counterclockwise pattern does not represent the precise mirror image of the diurnal branch. This mirror image would have to go through 0° (left ordinate) at 24 hours TLT (abscissa), but the scores are displaced to the left of this theoretical line. In other words, the birds bore somewhat farther to the right than expected. This deviation is unexplained. More experiments are clearly needed to establish fully the response of birds to the various experimental and natural conditions.

SUMMARY

1. Three homing pigeons, directionally trained in a semi-automatically operating apparatus at Durham, N. C. (36° 00′ N; 78° 56′ W), were displaced to Barrow, Alaska (71° 10′ N; 150° 41′ W), around the summer solstice of 1962.

2. At "night" the birds allowed dichotomously for a clockwise and a counterclockwise movement of the sun. This is the first indication that individuals may follow the "bee pattern" as well as the "*Talitrus* pattern."

---

[2] Fish in the northern hemisphere have been found to follow the bee pattern (Braemer, 1959), while others (of a different genus) on the equator followed the *Talitrus* pattern (Braemer, W., and H. Schwassman, 1963. Vom Rhythmus der Sonnenorientierung am Aquator (bei Fischen). *Ergebn. Biol.*, **26**: 253–258).

LITERATURE CITED

Braemer, W., 1959. Versuche zu der im Richtungsgehen der Fische enthaltenen Zeitschätzung. *Verh. Dt. Zool. Ges. Münster*, **1959**: 276–288.

Braemer, W., 1960. A critical review of the sun-azimuth hypothesis. *Cold Spring Harbor Symposium*, **25**: 413–427.

Birukow, G., 1960. Innate types of chronometry in insect orientation. *Cold Spring Harbor Symposium*, **25**: 403–412.

Hoffman, K., 1959. Die Richtungsorientierung von Staren unter der Mitternachtssonne. *Zeitschr. vergl. Physiol.*, **41**: 471–480.

Lindauer, M., 1957. Sonnenorientierung der Bienen unter der Äquatorsonne und zur Nachtzeit. *Naturwiss.*, **44**: 1–6.

Mittelstaedt, H., 1963. Bikomponenten-Theorie der Orientierung. *Ergebn. Biol.*, **26**: 253–258.

Papi, F., and J. Syriämäki, 1963. The sun-orientation rhythm of wolf spiders at different latitudes. *Arch. Ital. Biol.*, **101**: 59–77.

Pardi, L., 1960. Innate components in the solar orientation of littoral amphipods. *Cold Spring Harbor Symposium*, **25**: 395–401.

Schmidt-Koenig, K., 1961. Die Sonnenorientierung richtungsdressierter Tauben in ihrer physiologischen Nacht. *Naturwiss.*, **48**: 110.

Schmidt-Koenig, K., 1963. Sun compass orientation of pigeons upon equatorial and transequatorial displacement. *Biol. Bull.*, **124**: 311–321.

FLASH PATTERNS IN JAMAICAN FIREFLIES

H. H. SELIGER, J. B. BUCK, W. G. FASTIE AND W. D. McELROY

McCollum-Pratt Institute, Department of Biology, and Department of Physics, The Johns Hopkins University, Baltimore 18, Maryland, and the Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland

In most lampyrid fireflies the male flashes spontaneously in flight, whereas the female ordinarily remains at rest and flashes only in response to the flash of a male. It is well known that the flashing of male fireflies varies from species to species. The light differs in color, peak intensity and kinetics of emission (Harvey, 1952; Buck and Case, 1961; Seliger *et al.*, 1964) but the sequence and timing of flashing appear to be constant and species-specific. McDermott (1914) was the first to attempt to quantify these latter differences. The same notation was used by McDermott and Buck (1959) for their visual observations on 23 Jamaican species. In the latter paper the authors note that some species show variation in their flash patterns but add that the qualifications should not be allowed to obscure the really remarkable intraspecific constancy of the flash patterns, enabling the observer to recognize many of the species in free flight. Barber and McDermott (1951), in fact, used the visually observed flash patterns as an integral part of their attempt to sort out the North American species of *Photuris*. Visual observation cannot yield precise descriptions of flash contour and fine structure. These details are of particular importance, not only because they may serve as a more precise method of species identification, but because of their implications in regard to the ability of the photocyte and nervous system to control the underlying bioluminescent reaction. In the present work we therefore undertook to record the flashing by quantitative electronic means. Jamaica was chosen as the locale for operation because it provides a unique combination of the variety of firefly species characteristic of the moist tropics and terrain for field work which is both remote from artificial lights and safe for personnel. Furthermore, as already noted, there already exists a solid foundation of the taxonomic and histological work which is prerequisite for interpreting the physical data.

Material and Methods

Since fireflies usually flash spontaneously only during flight it was necessary to devise a photometer suitable for use in the field. In addition to the requirement of portability and humidity resistance of the electronic components the following requirements had to be met:

Signal-to-noise sensitivity

Coblentz (1912) reported the candlepower of the firefly flash to vary from 1/50 candela to 1/400 candela, with the predominating values being around 1/400 candela. While it is not proper to assess non-blackbody light intensities in photometric units of candelas we can make some estimates of the number of photons emitted per second by a "1/400 candela" firefly flash. One candela emits 4π lumens in all directions and the least mechanical equivalent of light is 0.00147 watts/lumen. This therefore corresponds to the emission of 4.6×10^{-5} watts, or at 5560 Å, the wave-length of maximum photopic luminus efficiency, to approximately 10^{14} photons per second. Firefly light therefore was the order of magnitude of the peak light intensity to be measured. At a distance of 15 feet the illumination would be 0.11×10^{-4} foot candles or 0.12×10^{-7} lumens/cm.^{2}. Moonlight, either direct or diffusely reflected, was to be avoided and ideally measurements were planned during the dark phase of the moon and in a direction pointing toward the dark sky or the vertical underbrush rather than directly down toward the grass. In addition there is the strong 5577 Å line of O_I present in the night sky.

With a phototube cathode area of 2 cm.^{2}, a peak illumination of 0.11×10^{-4} foot candles corresponds to a peak incident intensity of 10^8 photons per second. The dark noise of the phototube at maximum gain was equivalent to about 4×10^5 incident photons per second. This reasonably large signal-to-noise ratio permitted a 5440 Å interference filter, with a half width of 100 Å, to be used with the phototube. This permitted only a narrow green portion of the firefly emission spectrum to be detected, markedly reducing the signal-to-noise ratio. Furthermore this effectively reduced the contribution due to the 5577 Å O_I line. Under these conditions of filtering, an incident intensity of 2×10^8 photons/sec.-cm.^{2} of "pseudo-firefly" light, obtained by a method to be described in a later section, produced a signal equal to six times the phototube noise.

Acceptance angle

The firefly flash frequency is quite commonly as low as 10/minute and the flight velocity may easily be 2–5 feet per second. It is thus clear that the instrument should be able to monitor a radius of 20–30 feet if there is to be any reasonable chance of recording a sequence of three to four consecutive flashes from one individual. The usual firefly flight pattern is roughly straight and roughly parallel to the ground, but not sufficiently so that it is possible to predict exactly where, in the darkness, each successive flash of a given specimen will occur. Accordingly, the photometer needs to have a wide acceptance angle. This wide aperture, however, means background trouble from the general sky light. Fortunately this is less troublesome in the tropics than in regions with a long twilight.

In the resultant instrument a ½-inch diameter phototube (Dumont No. KM 2332) was used as the detector, gasket-sealed in a dural cylinder 8 cm. in diameter and 20 cm. long. By means of suitable diaphragms and collimators the phototube response was made flat over an acceptance solid angle of one steradian.

Time resolution

Even visually it is apparent that some species have a high-frequency flicker superimposed on the primary flash (*e.g., Pyractomena lucifera* and *Photuris* *pennsylvanica* in McDermott, 1914, and *Photinus ceratus, P. commissus* and *P. evanescens* in McDermott and Buck, 1959).

Further, half-rise time may be as little as 25 msec. (Brown and King, 1931; Snell, 1932; Alexander, 1943; Buck and Case, 1961). Accordingly, good time resolution is important in the photometer. The phototube electronic circuits are essentially the same as those used in previous measurements of marine bioluminescence (2, 3). A D.C. amplifier with a flat response up to 1000 c/s was used, together with a Sanborn two-channel Model 321 chart recorder, the latter having a flat response up to 100 c/s. The photometer unit, containing the phototube and the transistorized amplifier circuits, was connected to the control box through 15 feet of ¼-inch diameter flexible neoprene-covered cable.

Absolute photon calibration

The photometer was calibrated absolutely in photons/sec.-cm.^{2} in the following way: A National Bureau of Standards color temperature standard lamp was set up in combination with an empirically adjusted glass filter combination so that the resultant transmitted light had a spectral distribution very close to that of *Photinus pyralis*. This "pseudo-firefly" emission was obtained with the lamp operated at a color temperature of 2854° K and a composite filter combination consisting of Corning filters 1–69, 3–71, 3–76 and 4–96. Two centimeters of water in a Pyrex glass cell were used to absorb infrared energy. Using a thermopile, previously standardized with a National Bureau of Standards radiation standard lamp, the photometer was calibrated by means of a direct substitution technique.

The primary standardization of blackbody emission is based on energy emission. However, all reactions in photobiology are quantum phenomena and in any studies one is concerned with the number of photons involved. The steps in the conversion from the spectral distribution of firefly light and from the measurement of energy by the thermopile to photon flux are not immediately obvious. If the relative spectral distribution of *Photinus pyralis* bioluminescence is given by $f(\lambda)$, and the energy of a photon of wave-length in Angstroms is given by

$$E_\lambda = \frac{1987}{\lambda(\text{Å})} \times 10^{-18} \text{ Joule,}$$

the average energy of the *P. pyralis* emission is given by

$$\langle E \rangle = 1987 \times 10^{-18} \frac{\int f(\lambda)/\lambda \, d\lambda}{\int f(\lambda) d\lambda} \text{ Joule,}$$

where the integration is performed over the entire emission spectrum. Therefore, if W is the energy flux in watts/cm.^{2} measured by the thermopile at a fixed distance from the "pseudo-firefly" source, the photon flux is given by

$$I = \frac{W}{\langle E \rangle} \text{ photons/sec.-cm.}^2$$

At this same fixed distance but with an attenuation filter the photometer response was measured so that any scale reading could be converted to incident photons/sec.-cm.^{2} effectively emitted by *P. pyralis.* The fact that other fireflies have slightly different spectral distributions affects the accuracy of the measurements since each spectral distribution has a slightly different value of $<E>$. However, the errors introduced by assuming that all fireflies examined have a *P. pyralis* emission spectrum is of the order of 10–20% and is well within the uncertainties due to measurements of distances in the field during a flashing event. Even these errors can be corrected for if necessary since, as can be seen, I is inversely proportional to $<E>$.

From the absolute calibration and the complete flashing record it is also possible to obtain the total number of photons emitted per firefly flash.

Because of the precipitous jungle-covered terrain, roads provided almost the only localities flat and open enough for repetitive measurements. The species studied were beetles of the lampyrid genera *Lecontea, Photinus, Photuris* and *Diphotus,* and the elaterid bettle *Pyrophorus.* Except for *Lecontea gamma* and *Photinus commissus,* which were studied on Castle Hill, about a mile northwest of Long Bay, the records were made at an altitude of about 750 feet, on a remote section of the Ecclesdown Road, that runs through the foothills of the John Crow Mountains (Portland Parish). Fireflies were chosen that were flying along a straight stretch of the road. In making records one investigator attempted to keep the phototube directed at and close enough to the chosen firefly, a second monitored high voltage to the phototube of the photometer and operated the recording meter, and the other participants hovered nearby with insect nets, ready to capture the specimen as soon as the recordings were completed or the insect showed signs of flying off the road. As soon as a specimen was captured it was put in a vial for later identification from the key of McDermott and Buck, and given a number corresponding to that of the record chart.

RESULTS

Representative records of flashing behavior are presented in Figures 1 through 5. In viewing these it must be kept in mind that although the time scale (abscissa) is accurate, light intensity varies with distance from specimen. Attention is directed particularly to the following points:

A. *General flash patterns.* At the outset it is to be recalled that fireflies that have a light organ structure involving a regularly arranged tracheal supply, and tracheal end-cells (*Photinus, Photuris*) typically produce short sharp flashes, whereas those lacking end-cells (*Diphotus, Pyrophorus*) emit their light as long lingering glows (Buck, 1948). Among flashing-type lampyrids, we find luminosity in flight to vary from the single and usually homogeneous flashes of *Photinus melanurus* (Spec. 91, 20 Figure 3) and *P. leucopyge* (Spec. 5, 12 Figure 2), sometimes delivered with remarkable regularity, through the compound or flickering flashes of *Lecontea gamma* (Spec. 72 Figure 1), *Photinus evanescans* (Spec. 110B Figure 1, 125 Figure 1, 130 Figure 1), *P. gracilobus* (Spec. 132, 108 Figure 1), *P. lobatus* (Spec. 42 Figure 3) and *Photuris jamaicensis* (Spec. B Figure 4),

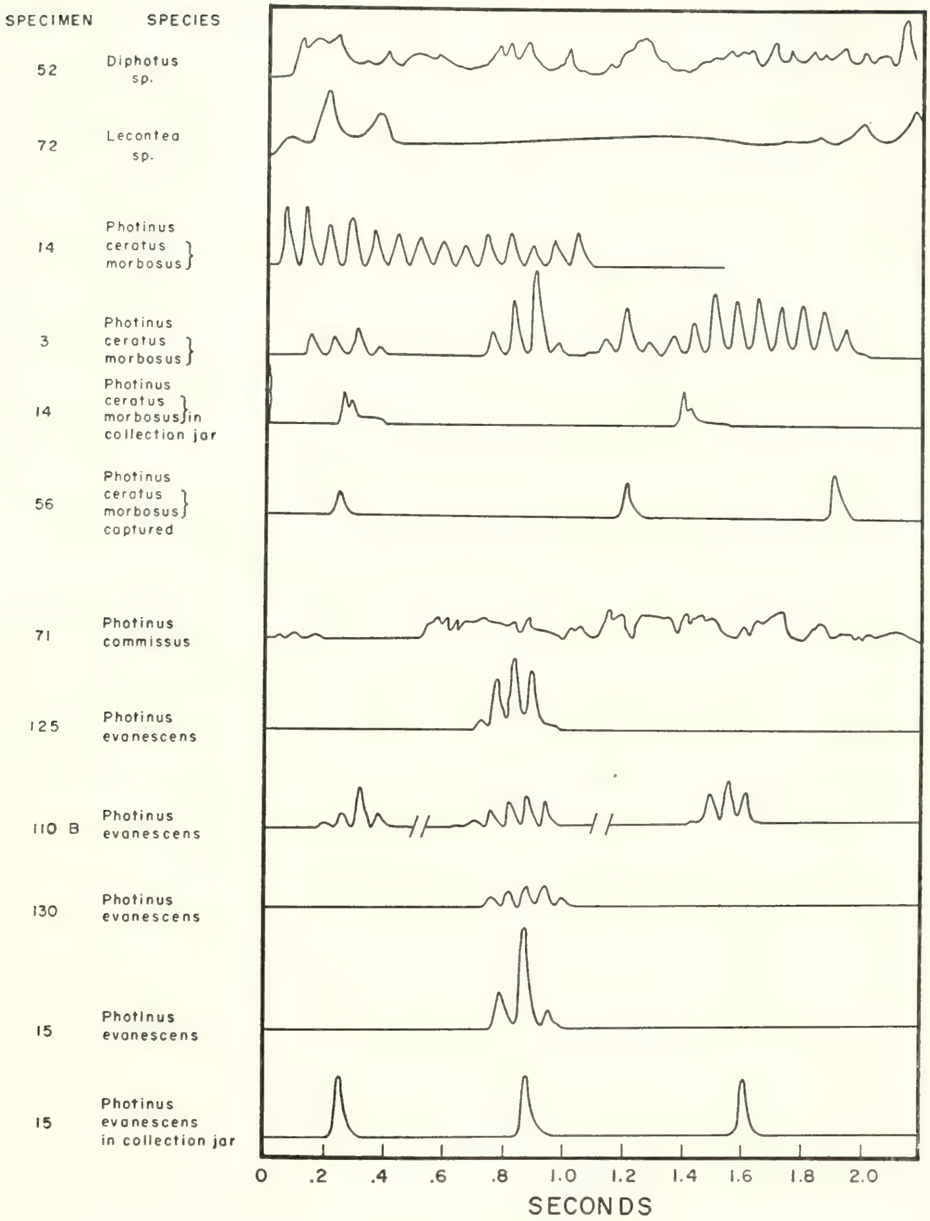


FIGURE 1. Direct tracings of field recordings of firefly flash patterns for *Diphatus* sp., *Lecontea* sp., *Photinus ceratus-morbosus, Photinus commissus* and *Photinus evanescens.*

to the complex pattern of *Photinus ceratus-morbosus*, in which two short flickers and a long flicker are grouped together (Spec. 3 Figure 1).

In contrast, the glowing types of firefly, *Diphotus sp.* (Spec. 52 Figure 2) and *Pyrophorus plagiophthalamus* (Spec. C, D, E, F, G Figure 5), showed light-production of the greatest irregularity. This unexpected finding (the light of both insects appears quite steady to the eye) will be discussed below. The causation of the similar record made by a flying female of *Photinus commissus* (Spec. 71 Figure 1) will be considered at the same time, but the phenomenon is not entirely surprising in view of the visual observation that female lampyrids, on the rare occasions when they do take wing, not infrequently show an irregular

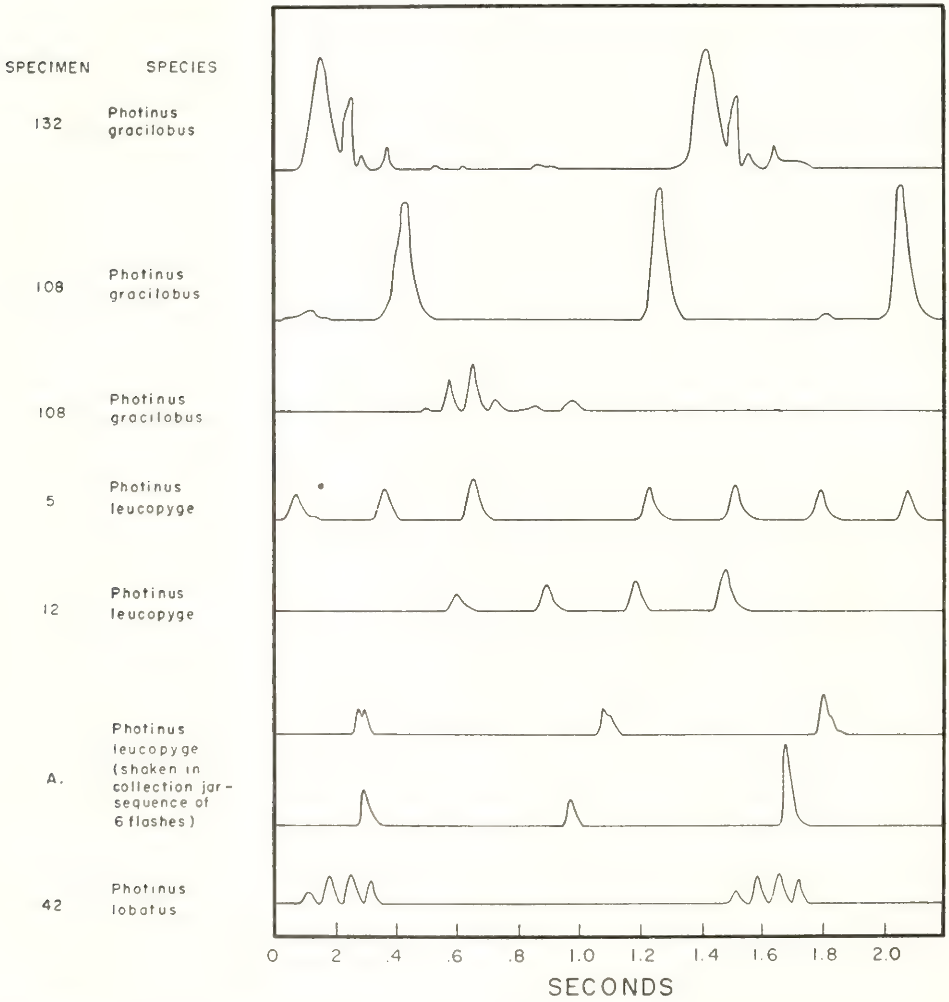


FIGURE 2. Direct tracings of field recordings of firefly flash patterns for *Photinus gracilobus*, *Photinus leucopyge* and *Photinus lobatus*.

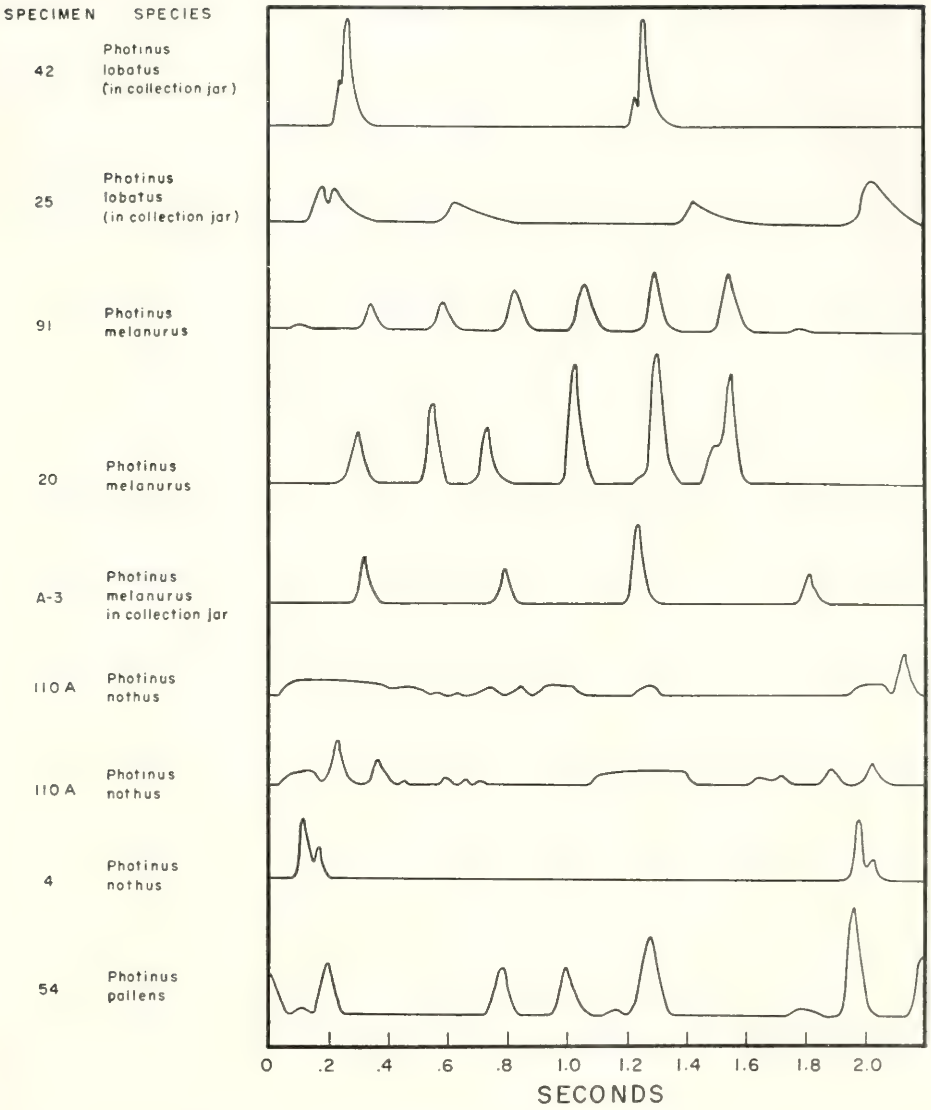


FIGURE 3. Direct tracings of field recordings of firefly flash patterns for *Photinus lobatus*, *Photinus melanurus*, *Photinus nothus* and *Photinus pallens*.

glow (*e.g.*, see remarks of Buck and Case, 1961, in reference to a North American photurid). It is likewise not wholly unexpected to find similar irregular glowing between the flashes of occasional flying males, for example *Photinus gracilobus*, (Spec. 132 Figure 2) and *P. pardalis*, (Spec. 129 Figure 4), since such "intercalated flashlets" were seen in several species by McDermott and Buck. However, records for Spec. 71 (Fig. 1) (*Photinus evanescens*) and Spec. 110A

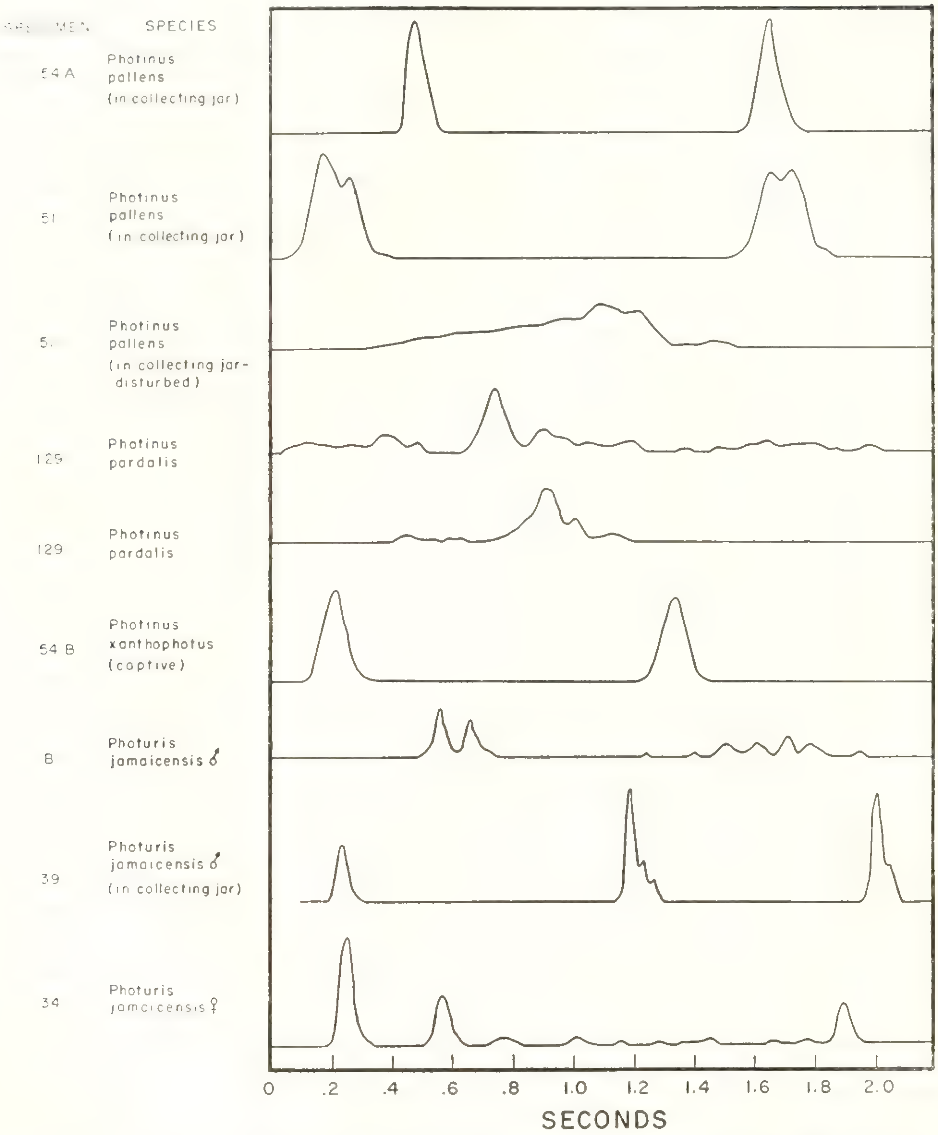


FIGURE 4. Direct tracings of field recordings of firefly flash patterns for *Photinus pallens, Photinus pardalis, Photinus xanthophotus* and *Photuris jamaicensis.*

(Fig. 3) (*P. nothus*) must be regarded as definitely atypical in their irregularity and paucity of clearly defined major flashes.

B. *Flashing in captivity.* As noted also by McDermott and Buck, there is often a pronounced difference between the flashing of male lampyrids in flight and in captivity. In general the change is in the direction of producing single flashes where the flight pattern is compound—for example Spec. 56 (Fig. 1) *vs.* Spec.

14 (Fig. 1) (*P. ceratus morbosus*), Spec. 15 (Fig. 1) *vs.* Spec. 110B (Fig. 1) (*P. evanescens*) and Spec. 25 (Fig. 3) *vs.* Spec. 42 (Fig. 3) (*P. lobatus*)—which corresponds to the field observation that fireflies at rest on bushes tend to flash singly, and at irregular intervals. However, we also recorded instances in which the captive animal produced a more complex flash than typical for flight—*e.g.,* Spec. 51 (Fig. 4) *vs.* Spec. 54A (Fig. 4) (*P. pallens*), Spec. 39 (Fig. 4) *vs.* Spec. B (Fig. 4) (*Photuris,* male) and Spec. 50 (Fig. 5) *vs.* Spec. 34 (Fig. 4) (*Photuris,* female). Unfortunately we did not record the amount of mechanical stimulation (shaking or jarring), if any, needed to induce flashing, so nothing

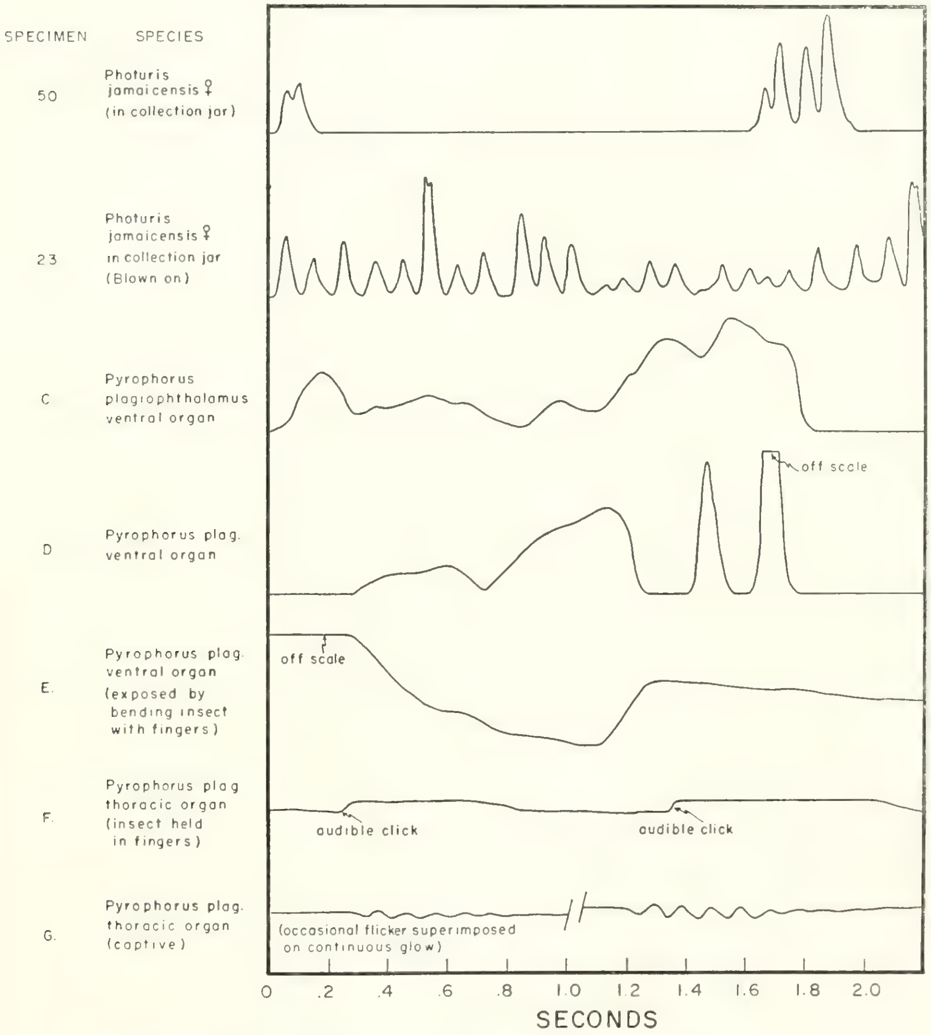


FIGURE 5. Direct tracings of field recordings of firefly flash patterns for *Photuris jamaicensis* and *Pyrophorus plagiophthalamus.*

can be said about the conditions for neural stimulation of flashing during flight *vis à vis* quiescence. It is, however, clear that the insects have the power of varying their flash pattern to a considerable extent.

C. *Variability and its taxonomic implications.* McDermott and Buck reported significant differences in flash patterns within certain species in regard to populations from different localities—*e.g.*, lowland *versus* mountain—and even occasionally between individuals from the same population. The present photometer records confirm this impression. It appears, therefore, that while the flash pattern has no absolute significance as a criterion of identification it can be a valuable supplement to the standard taxonomic characters which are themselves no less variable (Buck, 1942). A case in point is the species complex, *Photinus ceratus-morbosus,* erected as separate species by Barber (1941), redescribed by McDermott and Buck but without achieving a really sharp separation, and not separable in our present sample on the basis of either morphology or flashing behavior. Nonetheless our general experience has been to confirm solidly a specific individuality of many of the flash patterns and a pragmatic value in identification of the same order of consistency and usefulness as bird calls have for the ornithologist.

D. *Relation to visual records.* There is good agreement between our present photometer records and the visual observations of McDermott and Buck. In instances such as *P. evanescens, P. nothus, P. ceratus-morbosus* and *Photuris,* the eye recognizes the "flickering" or "twinkling" nature of minute light sources of low absolute intensity fluctuating of the order of 15 times per second. It is interesting, however, that the eye judges the variation in intensity between peak and trough to be very minor—a mere ripple in a plateau level of luminescence (McDermott and Buck, Fig. 1)—whereas the photometer shows the individual peaks of the compound flashes to be very well separated, intensity falling nearly or quite to extinction in the troughs.

In certain instances our records show the eye to have been at fault. The inadequacies were of two main kinds. First, some real twinkles were not resolved. For example, the short four-peak forerunners of the main 12–14-peak twinkle of *P. ceratus-morbosus* were seen as single flashes, dimmer than the main twinkle (see also McDermott and Buck, *ceratus* type 3c). This is strange in view of the success in resolving visually the apparently similar twinkles of *P. evanescens* (Spec. 110B, 125 Fig. 1), particularly since McDermott and Buck reported a twinkle sometimes so rapid as to look like a single flash or glow from a distance. Similarly the compound flash of *P. gracilobus* (Spec. 132 Figure 2) looks single. In the latter instance the failure to discriminate the separate peaks could well be due to the dominant intensity of the first and the incomplete extinction between the sub-peaks.

The display of *P. lobatus* (Spec. 42 Figure 3) is at variance with the single flash given by McDermott and Buck as typical of the lowland variety of this species. It does agree with the twinkle reported for the high-mountain form in summer observations, suggesting the interesting possibility of a migration to lower altitudes during the "winter" season; but unfortunately in the hurly-burly of recording from and capturing Spec. 42 we failed to note what was the visual impression of his flash. In this connection, incidentally, the triple twinkle of *P.*

ceratus-morbosus is also the high-altitude variant of this species (*ceratus*) according to McDermott and Buck.

In the second and more interesting discrepancy between visual and photometer records, the eye sometimes saw twinkles where the photometer saw none (*e.g.*, *P. leucopyge*). This is not as isolated a discrepancy as may appear because McDermott and Buck specifically note ". . . possibly a high-frequency flicker . . ." for three species which they reported as having a single flash (*Photinus lewisi*, *P. naevus* and *P. synchronans*). To add to the physiological interest of the question it is the strong impression of one of us (J. B.) that illusion of flicker is much stronger when a "flash" is seen in peripheral vision. The question is presently under separate investigation.

E. *Parameters of flash control.* The records of flashing indicate some of the potentialities of the mechanism controlling luminescence. For example, we see that the neural pacemaker that times the individual flashes during flickering can fire with remarkable regularity and at frequencies ranging from 10 to 18 per second in different species (Spec. 4 Figure 3, 23 Figure 5; 14, 3 Figure 1; 42 Figure 3; 125, 110B, 130 Figure 1). Similarly the kinetics of the individual flashes show that the neuroeffector control of the individual photocytes must be of a high order since the many thousand cells comprising the lantern can all complete their cycles of activity within 50–100 msec. Though, as mentioned earlier, the relative intensity of major flash episodes may be influenced by changing distance of the insect from the photometer, this factor should have little effect on records on the high frequency flashing within episodes of twinkling. Hence, the apparently consistent differences in relative intensity of the individual peaks within the flicker of *P. evanescens* (Spec. 125, 110B, 130 Figure 1) and *P. gracilobus* (Spec. 132 Figure 2), for example, probably represent a valid second order of species-specific neuroeffector programming of luminous emission. On the other hand, the intensity fluctuations within the long twinkles of *P. ceratus-morbosus* (Spec. 14, 3 Figure 1) and the *Photuris* female (Spec. 23 Figure 5) show that the emission is capable of spontaneous variation.

F. *Implications of variation in glow level.* Intensity fluctuations during long-continued luminescence are of special interest. Forms such as *Diphotus*, which lack the ability to produce a sharp brief flash, are nevertheless unexpectedly found to have variations in intensity superimposed on their continuous luminescence (Spec. 52 Figure 1). The fact that these variations are not detected by the eye is probably due to their small magnitude relative to the continuous luminescence and to the small change between successive peaks. However, the frequency of fluctuation equals or exceeds that seen in species able to produce concerted flashes. A clue to a possible mechanism is provided by analogy with the dim luminescence that is occasionally seen between the successive flashes of the usual lampyrid pattern. Microscopic examination of the lantern surface shows that this light is sometimes due to a generalized dull steady glowing of the luminous tissue, but sometimes also to numerous sparkling points, flashing on and off briefly and irregularly. This punctate "scintillation," studied at about 100 diameters magnification by Case and Buck (1963) in lanterns irrigated with eserin solution, was attributed to both single photocytes and small aggregations. This suggests, therefore, that both the irregular luminescence recorded from some individuals of flashing-type fireflies (*e.g.*, *P. commissus*, Spec. 71 Figure 1; *P. gracilobus*, Spec. 132 Figure 2; *P. nothus*, Spec. 110A Figure 3 and *P. pardalis*, Spec. 129 Figure 4), and that recorded from the glowing *Diphotus*, could be due to asynchronous and sporadic firing of small aggregations or photocytes.

The luminescence of *Pyrophorus* presents an especially interesting problem. Both the dorsal thoracic and ventral abdominal organs in this remarkable beetle, in captive specimens, seem to glow absolutely steadily, and nothing resembling flashing was ever seen in the field. The recordings made from individuals in flight (*i.e.*, from the abdominal organ) typically show great, though slow, fluctuations in intensity (Spec. C, D, E Figure 5). These are readily explained in terms of the great speed and erratic course of the insect's flight. (The two apparent flashes near the end of Spec. D may possibly be an unexplained exception but even these seem readily interpreted as "tracking errors" of the operator in his frantic efforts to keep the photometer pointed at the specimen.) In any case there is no indication of fine structure in rate of change of intensity, nor is there, sometimes, in the glowing of the thoracic organ in captive animals (Spec. F Figure 5). However, in some instances the photometer does detect a rapid, regular, small-amplitude pulsation superimposed on the continuous glow (Spec. G Figure 5). Harvey (1931), by the use of a string galvanometer, recorded what is apparently the same phenomenon though with a somewhat lower frequency (6 per second at first, decreasing in 15 seconds to 2.5 per second). In any case, this apparently well coordinated control, approaching in frequency the best achieved by fireflies with tracheal end-cells, poses an important question for future investigation.

TABLE I

Light intensity emitted by various fireflies

Species	Peak Photon Intensity	Total Photons per Flash
Photinus ceratus-morbosus	14 × 10^{12}/sec	0.4 × 10^{12}
Photinus lobatus	17.5	1
Photinus pallens	18	2.4
Photinus xanthophotus	108	10
Photinus jamaicensis	52.5	3.4
Photinis jamaicensis	28	0.7
Photinus jamaicensis (blown on—excited)	112	2.8
Pyrophorus plagiophthalamus (thoracic organ)	100	Emits continuously

G. *Absolute photon emission.* The absolute photon efficiency of the photometer was determined for the North American firefly, *Photinus pyralis*, by the method described previously. Using this calibration it was possible to determine both the maximum light intensity and the total light quanta emitted per flash for a number of Jamaican species. The specimen was held or agitated in a glass jar at a measured distance from the phototube in a dark room. The results are presented in Table I. It is surprising that there is only about an 8-fold variation in peak intensity, not obviously correlated with size of lantern, and that there is no distinction in this respect between glowing-type fireflies (*Pyrophorus*) and flashers. The total photon emission should reflect the mass of photogenic tissue and flash duration, and would be expected to vary widely.

The authors would like to thank Mr. J. Veise and Mr. W. Biggley for their assistance in the construction of the firefly photometer, and Dr. J. Lee for his assistance in the photon intensity calibration. We wish to express our special gratitude to Drs. C. Bernard Lewis and Thomas Farr of the Institute of Jamaica for their invaluable aid in quartering and supplying the expedition and to Mr. and Mrs. W. Vandivert, Mr. F. A. McDermott and Mr. William S. Glidden for generous help with various aspects of the field work. This work was supported by The National Science Foundation, The National Institutes of Health and The Atomic Energy Commission, Division of Biology and Medicine.

SUMMARY

1. A portable photometer has been devised, permitting recording of flashes of flying fireflies in the field under natural conditions.

2. Various Jamaican fireflies typically emit their light according to one of the following patterns: (a) long-continued flow, usually fluctuating in intensity, (b) single concerted flashes of 75–100-msec. duration, delivered at regular intervals of 2 to 6 or more seconds, (c) one or more twinkles or flickers consisting of 4–20 or more short flashes at frequencies of 10–18 per second, delivered every few seconds.

3. In captivity a given species usually gives a simpler type of flash than when in flight.

4. The flash patterns are highly constant and are characteristic of particular species, though not completely invariant.

5. The photometric records show some visual impressions of firefly flash type to be in error, particularly in the detection of flicker.

6. The photometric records show that the photogenic control mechanism is capable of inducing peak flash luminosity or of extinguishing the light in periods of the order of 30–60 msec.

7. Unexplained high-frequency fluctuations in intensity of glowing are provisionally attributed to uncoordinated firing of photocytes in small groups.

8. Data for absolute photon emission are given for six species.

LITERATURE CITED

ALEXANDER, R. S., 1943. Factors controlling firefly luminescence. *J. Cell. Comp. Physiol.*, **22**: 51–71.

BARBER, H. S., 1941. Species of fireflies in Jamaica. *Proc. Rochester Acad. Sci.*, **8**: 1–13.

BARBER, H. S., AND F. A. McDERMOTT, 1951. North American fireflies of the genus *Photuris*. *Smithson. Misc. Coll.*, **117**: 1–58. Publ. 4051.

BROWN, D. E. S., AND C. V. KING, 1931. The nature of the photogenic response of *Photuris pennsylvanica*. *Physiol. Zoöl.*, **4**: 287–293.

BUCK, J. B., 1942. Problems in the distribution and light organ structure of Jamaican lampyrid fireflies. *Year Book, Amer. Philos. Soc. for 1942, pp. 124–129.*

BUCK, J. B., 1948. The anatomy and physiology of the light organ in fireflies. *Ann. N. Y. Acad. Sci.*, **49**: 397–482.

Buck, J. B., and J. F. Case, 1961. Control of flashing in fireflies. I. The lantern as a neuroeffector organ. *Biol. Bull.*, **121**: 234–256.

Case, J. F., and J. B. Buck, 1963. Control of flashing in fireflies. II. Role of central nervous system. *Biol. Bull.*, **125**: 234–250.

Coblentz, W. W., 1912. A physical study of the firefly. Carnegie Inst. of Washington. Pub. No. 164, 1–47.

Harvey, E. N., 1931. Photocell analysis of the light of the Cuban elaterid beetle, *Pyrophorus*. *J. Gen. Physiol.*, **15**: 139–145.

Harvey, E. N., 1952. Bioluminescence. Academic Press, New York.

McDermott, F. A., 1914. The ecological relations of the photogenic function among insects. *Zeitschr. wiss Insekt. Biol.*, **10**: 303–307.

McDermott, F. A., and J. B. Buck, 1959. The lampyrid fireflies of Jamaica. *Trans. Amer. Entomol. Soc.*, **85**: 1–112.

Snell, P. A., 1932. The control of luminescence in the male lampyrid firefly *Photuris pennsylvanica*, with special reference to the effect of oxygen tension on flashing. *J. Cell. Comp. Physiol.*, **1**: 37–51.

Seliger, H. H., J. B. Buck, W. G. Fastie and W. D. McElroy, 1964. The special distribution of firefly light. *J. Gen. Physiol.* (submitted)

STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES. IV. THE TIME SEQUENCE OF PHOSPHORYLATION CHANGES IN VIVO[1]

HENRY T. YOST, JR., ROBERT M. GLICKMAN AND LAURENCE H. BECK

Department of Biology, Amherst College, Amherst, Mass.

Previous work has demonstrated that mitochondrial oxidative phosphorylation is more sensitive to uncoupling by ionizing radiation when the mitochondria are exposed *in vivo* than when they are irradiated *in vitro* (van Bekkum, 1957; Yost and Robson, 1959; Benjamin and Yost, 1960). These findings are consistent with the reports of the effects of irradiation on the phosphorylating mechanism by a number of other authors and have led to the hypothesis that the action of radiation in producing the *in vivo* effect is indirect and probably moderated by radiation-induced alterations of the hormonal balance of the organism.

The main purpose of the studies reported in this paper was to determine the time sequence of inactivation and recovery of the phosphorylating mechanism. The data presented by Benjamin and Yost (1960) were insufficient to permit conclusions about the time of onset and the time of repair of damage. This is particularly evident since van Bekkum (1957) had reported activation as early as eight hours post-irradiation and Noyes and Smith (1959) reported inactivation of a short-term nature at one hour post-irradiation. Consequently the pattern of inactivation was investigated, using our techniques, to determine whether the effect studied in this laboratory was the same as that studied by others. Furthermore, it was possible to correlate the damage to the phosphorylating mechanism with damage to radio-sensitive cells and tissues for which the time sequence is well established, particularly the spleen (Metcalf, Blandaw and Barnett, 1950) and the circulating lymphocytes (Jacobson, 1954).

In the second place, studies of the time sequence of inactivation can be used to further test the hypothesis of indirect action of radiation on the phosphorylating mechanism. Experiments were performed in which the time of onset and the time of repair of damage were determined for total-body and partial-body exposure. For example, rats whose heads were shielded were compared with those whose entire body had been exposed, on the proposition that, if the effect was largely moderated by hormonal changes originating in the pituitary, head-shielded rats would show a reduced response. Such experiments have the additional value of providing evidence to assess whether "resistance" to irradiation is a function of initial resistance to damage or of the rate of regeneration of damaged cells and tissues.

---

[1] This work was supported in part by a grant (RH-82) from the Radiological Health Institute and a grant (C-6132) from the National Cancer Institute.

Finally, since previous studies have often given results in which the P:O values are relatively low, it seemed wise to reinvestigate the problem with techniques which would insure "normal" P:O values for control animals. Recently, reports by Thomson and Rahman (1962) and Thomson (1964) have suggested that the observed inactivation of phosphorylation by irradiation is an artifact of assay procedures which give low P:O values. Such an objection does not apply to this work, since the data presented have been collected in such a way that low P:O values are avoided. Although the technical problems raised by Thomson will be discussed elsewhere, it is significant to point out that the studies done in the present paper show that the times at which the assays were done with spleen mitochondria (Thomson, 1964) were such that no effect of irradiation would have been expected. Therefore, it is hoped that the publication of the time-sequence data in this paper will make possible better experimental design for investigation of this rather complex indirect effect of irradiation, in the future.

Materials and Methods

All experiments were carried out with young, male, albino rats weighing from 200 to 250 grams. Rats of the Sprague-Dawley or Wistar lines were used exclusively. With the exception of those rats assayed soon after irradiation, all rats were anesthetized prior to exposure with 0.04–0.05 mg. Nembutal per gram of body weight. At the times of assay, there was no residual effect of the anesthetic on phosphorylation. Those rats assayed up to eight hours after exposure were irradiated without anesthetic, in a small cage permitting little freedom of motion.

Shielding of the anterior abdomen was accomplished by covering the region (from the lower extremity of the rib case to beneath the stomach) with two inches of lead plate. (This method gave the same result as shielding the exteriorized spleen (Benjamin and Yost, 1960) and resulted in far fewer fatalities. However, since more than the spleen was shielded, this procedure may decrease the effectiveness of the radiation.) Head-shielding was performed in a similar manner, with the head removed to the edge of the field, as well as covered by lead. Measurements with a Victoreen dosimeter indicate that this method permits about 10% of the delivered dose to reach the shielded organs, since Co^{60} γ-rays are difficult to eliminate entirely. The rats were irradiated singly in all cases, and during the period between treatment and assay, they were fed Purina Lab Chow and kept supplied with water. In order to assure maximum uniformity, large numbers of rats were irradiated successively, caged together, and selected randomly for post-irradiation assay. Control rats were processed in exactly the same manner with the sole exception of exposure to radiation. All radiation was delivered from a 220-curie Co^{60} source, filtered with ½ inch of Lucite, at an intensity of 90 r/min. Since there is a slight lack of uniformity of dose resulting from the thickness of the animals, the dose was calculated for a plane passing through the pituitary and spleen of the rats.

One hour before sacrificing a group of rats, lymphocyte counts were made. Blood was obtained by cutting off one-eighth inch of the tail. The first drop was discarded in all cases where the state of the animal permitted. Cell counts were made at a dilution of 1:20, using a dilution fluid of 1% acetic acid and gentian violet. Replicate counts done with control blood samples indicated an error in counting of 10%.

The rats were sacrificed by a blow on the head and the spleens removed. Small sections of spleen tissue were taken from all experimental animals of any one run. (Since samples taken from the tip and from the mid-part of the control spleens indicated that the tissue was homogeneous, and since the small size of spleens from irradiated rats made it inadvisable to sacrifice much tissue for histological examination, sections were taken routinely near the tip of each spleen.) The tissue was fixed in Bouin's fluid for 12 to 15 hours, washed and dehydrated with alcohol, and embedded in paraffin. Sections were cut at 10 μ and stained with hematoxylin-eosin stains. Sections from spleens of the different animals within any one experiment were kept separate and compared, to determine the amount of variation existing in animals receiving the same treatment.

The bulk of the spleen tissue (remaining after the above procedure) was pooled from two or three rats and homogenized in cold, isotonic sucrose solution (5 ml. per spleen) containing 0.005 *M* disodium versenate. Mitochondria were isolated from the homogenate by differential centrifugation (Schneider, 1948), with the particulates collected at 9000 *g* and washed once. Routinely, mitochondria derived from control animals were suspended in 0.9 ml. sucrose-Versene and those derived from irradiated animals were suspended in 0.5 ml. for assay. The unequal dilutions were made necessary by the lower yield of mitochondria from irradiated animals (as measured by O.D.). If the preparation is too dilute, generation of ATP does not occur, regardless of the source of the mitochondria. After irradiation, the spleen of the rat undergoes extensive change and the yield of mitochondria is greatly reduced. To maintain an adequate concentration of mitochondria, it is necessary to use an *apparently* more concentrated preparation from the irradiated spleens, otherwise phosphate uptake does not occur. To obtain reproducible results, very concentrated preparations of mitochondria were used throughout the experiments. (It is important to note that the mitochondrial preparation cannot be too concentrated; the addition of excess amounts of mitochondria, over a wide range, makes no difference to the P:O ratio. It is only essential that a certain minimal concentration be maintained.) The technique used in this paper insures that P:O ratios greater than zero will be obtained in all experimental cases; however, it is important to note that we are measuring the ability of *surviving* mitochondria to phosphorylate and possibly over-estimating the ability of the cells from irradiated animals to generate ATP, since the number of mitochondria per cell may be greatly reduced (Detrick, Upham, Springsteen, McCandless and Haley, 1964).

Liver mitochondria were prepared by homogenizing approximately 2 grams of liver removed from a single rat. The yield of mitochondria from liver is very high, and consequently it was unnecessary to pool mitochondria. In the case of testis mitochondria, however, four testes were necessary (two rats) to provide a sufficiently large pellet of mitochondria to give even minimally acceptable P:O ratios. Thus, only in the case of liver mitochondria do the data represent findings derived from a single animal. Otherwise, any individual P:O value represents an "average" of the conditions in two or three rats.

Table I

Depression of spleen phosphorylation by 800 r, total-body.
Sprague-Dawley rats

Time after exposure	P:O ratio	(Range)	% Inactivation	No. runs	Histology*
0	1.9	(1.8—2.0)	0	3	normal, many large follicles
1 hr.	2.0	(1.8—2.4)	0	3	pycnotic nuclei
3 hrs.	1.8	(1.6—1.9)	5	3	
8 hrs.	1.4	(1.2—1.5)	26	5	follicles reduced, debris
24 hrs.	1.1	(0.7—1.6)	42	11	follicles absent
48 hrs.	0.9	(0.8—1.1)	53	4	follicles absent
3 days	1.2	(0.9—1.8)	37	8	follicles absent
5 days	1.3	(0.9—1.8)	32	8	
8 days	1.0	(0.5—1.7)	47	8	
14 days	1.0	(0.9—1.2)	47	4	follicles absent, new cells
30 days	1.4	(0.8—1.7)	26	4	follicles present but tiny
43 days	1.9	(1.8—2.0)	0	3	follicles regenerating
Control (ave.)	1.9	(1.3—2.4)	—	64	

* Refer to text for description.

The efficiency of respiratory energy conversion was measured by the P:O ratio, using the technique of Hunter (1955). Oxygen uptake was measured in a Warburg respirometer at 25° C. Readings were taken for 20 to 30 minutes, after a 5-minute equilibration period; satisfactory P:O ratios are obtained from concentrated spleen preparations, so long as at least 6 micro-atoms of oxygen are consumed within that time period. Succinate was used as the substrate, and the incubation medium was the same as described previously (Yost and Robson, 1959). In all cases, ½ ml. of mitochondrial suspension was added to each flask. Estimation of the remaining inorganic phosphorus was carried out by the method of Lowry and Lopez (Glick, 1949). All phosphate tests were run in duplicate.

Table II

Depression of spleen phosphorylation by 800 r, total-body.
Wistar rats

Time after exposure	P:O ratio	(Range)	% Inactivation	No. runs	Histology*
24 hrs.	1.2	(1.1—1.2)	33	3	follicles absent
4 days	1.1	(0.9—1.2)	39	4	
5 days	1.0	(1.0—1.1)	44	3	
6 days	1.1	—	39	3	follicles absent, new cells?
7 days	1.5	(1.3—1.6)	17	4	
8 days	1.7	(1.6—1.8)	6	6	tiny follicles, when present
11 days	1.8	(1.7—1.9)	0	3	follicles regenerating
Control (ave.)	1.8	(1.6—2.3)	—	26	

* Refer to text for description.

TABLE III

Depression of liver phosphorylation by 800 r, total-body. Sprague-Dawley rats

Days after exposure	P:O ratio	(Range)	% Inactivation	No. runs
2	1.2	(0.8—1.4)	33	5
4	1.4	(1.3—1.4)	22	5
6	1.5	(1.3—1.6)	17	5
8	1.6	(1.4—1.7)	11	5
10	1.7	(1.6—1.8)	6	5
12	1.8	(1.7—1.8)	0	5
Control (ave.)	1.8	(1.7—2.1)	—	30

RESULTS

Phosphorylation

The data in Tables I to VI are presented as average P:O values from all determinations at any particular time. Although individual control and experimental animals were processed simultaneously in each run, the amount of data presented in this paper is too extensive to permit individual experiments to be presented in tabular form, as has been done for other studies (Benjamin and Yost, 1960). For simplicity of presentation, control values from all experiments in any one table were averaged as a basis for comparison, rather than having the control average for each set of runs presented with the experimental. Inactivation is computed from the average P:O value for each time period compared to the overall average for the control. Values obtained in this way do not differ significantly from those obtained by presenting individual experiments (certainly the values for the inactivation are approximations at best). It is important to note that although there is a variation in the controls, whose range would appear to overlap that of the experimentals, at no time did experimental

TABLE IV

Depression of testis phosphorylation by 800 r, total-body. Sprague-Dawley rats

Days after exposure	P:O ratio	(Range)	% Inactivation	No. runs
2	0.7	(0.7—0.8)	42	5
4	0.8	(0.7—0.9)	33	5
6	0.9	(0.8—1.0)	25	5
8	0.9	(0.9—1.0)	25	5
10	0.9	(0.9—1.0)	25	5
12	1.0	(0.9—1.1)	17	5
14	1.1	(1.1—1.3)	8(?)	5
16	1.2	(1.1—1.3)	0	5
Control (ave.)	1.2	(1.1—1.3)	—	40

TABLE V

Depression of spleen phosphorylation by 800 r, anterior-abdomen shielded. Sprague-Dawley rats

Time after exposure	P:O ratio	(Range)	% Inactivation	No. runs	Histology*
24 hrs.	0.6	(0.6—0.7)	68	3	follicles absent
48 hrs.	0.8	(0.4—1.1)	58	3	follicles absent
4 days	1.1	(0.9—1.3)	42	3	
6 days	1.6	(1.5—1.7)	16	5	follicles absent, new cells
8 days	1.8	(1.6—2.0)	5	6	follicles regenerating
11 days	1.9	(1.7—2.0)	0	3	follicles recovered
Control (ave.)	1.9	(1.6—2.0)	—	23	

* Refer to text for description.

P:O values in fact overlap the controls within individual experiments, until values of 5% inactivation (or lower) were reached. Thus, throughout the tables, the results of individual experiments are in agreement with the averaged data as presented.

One of the great difficulties in handling data of this type is in the development of a test for the significance of differences. Where the number of tests is suffi-

TABLE VI

Depression of spleen phosphorylation by 800 r, partial-body. Wistar rats

Time after exposure	P:O ratio	(Range)	% Inactivation	No. runs	Histology*
		Anterior-abdomen shielded			
24 hrs.	1.4	(1.3—1.5)	22	3	follicles absent
48 hrs.	1.2	(1.1—1.2)	33	3	follicles absent, new cells?
4 days	1.2	(1.0—1.3)	33	4	follicles regenerating
6 days	1.6	(1.2—1.7)	11	5	
8 days	1.9	(1.8—2.0)	0	3	follicles normal
Controls (ave.)	1.8	(1.5—2.0)	—	18	
		Head Shielded			
24 hrs.	1.8	(1.6—2.0)	5	3	follicles reduced in size
5 days	1.8	(1.8—1.9)	5	3	follicles normal
8 days	1.9	(1.8—2.0)	0	2	normal
Controls (ave.)	1.9	(1.8—2.1)	—	8	

* Refer to text for description.

ciently great (a minimum of 6 and more favorably 10 runs), standard deviation values of approximately 0.15 are obtained. Thus, to get highly significant results it is necessary that the difference in P:O ratios between controls and experimentals be greater than 0.45. To be extremely cautious it is best to use the value 0.5. Thus, in Table I where the data are most extensive, it is clear that there is a significant difference between the depression which occurs at 24 hours, three days, five days, and 8 days, and the controls. By ordinary standard deviation analysis, however, no such significance could be demonstrated for many of the other lines of data. Therefore, taken individually such lines of data must be treated as suggestive of the pattern of inactivation in the organism, rather than as established differences in inactivation from the compared controls. On the other hand, since the pattern remains unchanged, and since the experimental values at no time overlap the control values (so long as inactivation has occurred), it seems permissible to lump the data for statistical treatment by Chi-square analysis. When this is done the difference between experimentals and controls is highly significant ($P < 0.001$). Thus, the data presented in this paper clearly indicate that inactivation of oxidative phosphorylation occurs after exposure to radiation and, taken as a whole, indicate that this alteration in the phosphorylating mechanism requires a certain minimum time to be expressed and a certain minimum time to be repaired. Within any table, however, differences between neighboring lines of data are likely to lack significance and must be treated merely as a probable pattern to the extent that they correlate with other types of damage which are readily observed in the organism.

Table I shows that the inactivation of phosphorylation by total-body irradiation is not permanent. In the interpretation of these data, it is necessary to remember that those rats assayed at later times are those which have survived the effects of the radiation. Therefore, it is hard to tell how much of the restitution is attributable to recovery and how much to initial radiation resistance. Unfortunately, it is impossible to assay the same rat at two different times. The sampling error is made clear by the range of P:O values. Even at 24 hours post-irradiation, there was *one* Sprague-Dawley preparation which *approached* normal values. In general, the P:O values cluster around the mean, but there are always a few which fall at the extremes. Part of this variation is the result of the difficulties normally associated with P:O determinations, but the existence of rats within the population which show greater resistance is a factor which cannot be ignored. By 48 hours after irradiation, no rats sampled show even near-normal phosphorylation. The examination of several hundred cases in various experimental series has shown this to be a consistent effect. Thus, any initial resistance to radiation must be a matter of the first hours. In general, it is evident that recovery is a more important process than is resistance, insofar as phosphorylative ability is concerned. This is further borne out by the data obtained from histological examination (see below), which indicate that the rats respond uniformly to a given dose of radiation. Taking all the tables together, it is clear that there is no evidence for resistance to the initial effects of the radiation, either insofar as the phosphorylating mechanism is concerned or with regard to the histology of the spleen itself.

The data presented in Table II indicate that the Wistar line recovers from the initial effects of the radiation much more rapidly than the Sprague-Dawley line. These experiments were originally conducted on the basis of a report (Boche and Bishop, 1950) suggesting that the Wistar strain of rats is more radiation-resistant than the Sprague-Dawley strain. Therefore, it seemed possible that there would be a difference in the damage done to the phosphorylation system by equal doses of radiation. It is clear that the initial effects of the radiation in both strains are the same. However, it is also clear that under the conditions of this experiment the Wistar rats recover the ability to phosphorylate much more rapidly than do the Sprague-Dawley rats. Unfortunately, attempts to demonstrate a difference in the radiation resistance of the two strains, under the conditions existing at this laboratory, have not met with success. In the survival studies, the initial resistance of the Wistar rats is higher than that of the Sprague-Dawley; that is to say, more deaths occur in the early days with the Sprague-Dawley rats than with the Wistar rats. However, by the end of 30 days both strains show approximately the same response to radiation. Consequently, it appears that the alteration in the phosphorylating mechanism cannot be correlated directly with survival. Comparison of Tables I and II makes it clear that the recovery pattern is not an artifact of selection procedures. The two lines consistently recover at different times and are, therefore, at different levels of phosphorylative ability at the same time.

TABLE VII

Depression of liver phosphorylation by 800 r, anterior-abdomen shielded. Sprague-Dawley rats

Days after exposure	P:O ratio	(Range)	% Inactivation	No. runs
2	1.3	(1.0—1.5)	28	4
4	1.2	(1.0—1.3)	33	4
6	1.4	(1.3—1.5)	22	4
8	1.8	(1.6—2.0)	0	4
10	1.8	(1.7—1.8)	0	4
12	1.8	(1.7—1.9)	0	4
Control (ave.)	1.8	(1.7—2.0)	—	24

The data presented in Tables III and IV indicate that the effect on phosphorylation is not limited to the spleen. In both the liver and the testis of Sprague-Dawley rats an initial depression of phosphorylation is found, which follows the pattern observed in spleen. Similarly, there is recovery, but the rate of recovery is considerably different in different organs. Thus, the liver recovers much more rapidly than the spleen or the testis, the testis being intermediate in its rate. The work of Benjamin and Yost (1960) has indicated that the action of γ-rays is modified by different glands in producing the effect on liver and on spleen. (The nature of the action on the testis is not certain at the present time.) It is clear that, whatever the cause of the initial damage, the major difference in response is in the recovery rate. The collected data of Tables I–IV suggest that there is a widespread depression of phosphorylative ability in the entire body at 24 to 48 hours after exposure to radiation.

The data presented in Tables V, VI and VII demonstrate the efficacy of anterior abdominal shielding in accelerating the recovery process. The spleens of Sprague-Dawley rats recover their ability to phosphorylate long before they would without the shielding (compare with Table I). In the Wistar line, the *acceleration* of the recovery process is not as marked but is none the less evident. Similarly, the liver recovers only slightly more rapidly, but the difference is interesting since the liver, itself, is not shielded. It can be seen that the Wistar strain shows very little effect of exposure to radiation so long as the head is shielded. It must be remembered, in considering these data, that approximately 10% of the radiation reaches the shielded area in these experiments. At the present time, working with gamma radiation, it is not possible for us to refine our shielding procedures.

Histology

It has been known for some time that exposure to radiation causes profound changes in the "white pulp" (hereafter called follicles) of the spleen (Metcalf, Blandaw and Barnett, 1950). It seemed logical to investigate such histological changes at the same time that assays of the ability of the spleen cells to carry on oxidative phosphorylation were being made. It was hoped to use these well-established changes in tissue organization as an internal control for the effect on phosphorylation. Since the histological changes reported here are in no way different (except sampling time) from those previously described by others (Jacobson, 1954; Metcalf, Blandaw and Barnett, 1950), only a brief description of the typical cases is given. A short summary phrase is included in the tables for ease of comparison.

The first evidence of radiation damage is pronounced nuclear pycnosis of the lymphocyte tissue of the follicles at about one hour after exposure. By 8 hours after exposure, the follicles have been reduced greatly in size, with a marked accumulation of cellular debris. At the end of 24 hours, the follicles have been reduced to such an extent that there is little evident follicular material. Even the cells closely surrounding the arterioles at the center of the follicles show marked pycnosis. There is a change in the number of cells in the red pulp as well (Metcalf, Blandaw and Barnett, 1950), but these were not studied in detail in the present work, as all histological changes in the spleen seem to follow the more striking pattern of the follicles. After 24 hours, the pattern of recovery varies with the type of exposure, dose, age, etc. The descriptions which follow are in general agreement with those reported elsewhere except in the time-sequence, which varies as a result of such differences.

The spleens of the Sprague-Dawley rats show marked follicular growth by the 40th day. Although the spleens have not yet returned to normal, there is no doubt that recovery is extensive by this time. Pycnotic cells are rare and follicle size is about one-half that of the control animals. At 11 days post-irradiation, the follicles show some sign of recovery (the process is initiated), but there are far fewer follicles and none are more than one-tenth the size of the controls.

By 30 days after exposure, the size of some of the follicles has returned to normal, although the number of follicles is still reduced. It is significant that no follicles with normal histological appearance were found in these rats up to 20 days after exposure.

Shielding of the spleen (anterior abdomen) has little effect on the initial histological effect of the radiation. Degeneration of the splenic follicles is as drastic, in most cases, although in a few cases some small areas of the major follicles (near the arteriole) remain. (The nuclei of such surviving cells show marked pycnosis.) The rate of recovery is remarkable. By four days after exposure, the Wistar rats show extensive regeneration, with the larger follicles approaching the normal condition; and by eight days after exposure, these rats have apparently recovered fully. Much the same is true of the Sprague-Dawley rats, although they were somewhat delayed when compared with the Wistar line.

The Wistar rats were studied after exposure with the head shielded. The initial histological effects were reduced but were evident nonetheless. It is clear that follicular changes are induced even under these conditions. However, recovery is complete by five days post-irradiation, in all cases, and at no time is the damage as extensive as when the head is exposed.

TABLE VIII

Lymphocyte counts after 800 r, total-body or partial-body exposure. Sprague-Dawley and Wistar rats

Time after exposure	Total-body S-D	%*	Anterior-abdomen shielded S-D	%*	Total-body W	%*	W Head shielded	%*
24 hrs.	3350	26	3930	31	4400	35	4030	33
3 days	—	—	—	—	3230	25	7200	57
4 days	4120	32	6400	50	—	—	—	—
5 days	—		—	—	7000	55	9770	77
7 days			6480	51	—	—	—	—
8 days	4670	37	7570	60	9000	71	16120	127
10 days	4170	33	9000	71	—	—	—	—
12 days	4800	38	10000	79	10000	79	18220	143
14 days	**	—	13200	104	—	—	—	—

* Based on average control value of 12700/mm^3.
** Extremely variable.

The histological changes reported above are associated largely (although not entirely) with changes in the lymphocyte population. Since the spleen represents the largest mass of lymphocyte tissue in the body, it seemed advisable to attempt to correlate the changes in spleen organization and physiology with changes in the circulating lymphocyte population. The results of cell counts are presented in Table VIII. Because of the variability of the counts (the standard error of some counts was as high at 1000), the data can be taken only as a guide to the general pattern of effect and recovery. However, the depression in lymphocyte number follows the same pattern observed for phosphorylation and tissue damage. Where comparable, these results are in agreement with those of others (Jacobson, 1954).

DISCUSSION

The results presented in this paper clearly demonstrate that oxidative phosphorylation is uncoupled by exposure of rats to whole- and partial-body irradiation. The techniques employed in this work were such that the P:O values of control animals are in agreement with the theoretical value (2.0), with the exception of testis mitochondria. Therefore, the suggestion of Thomson (1964) that the effect of irradiation on oxidative phosphorylation is an artifact of preparation resulting in low P:O values for the controls as well as the experimentals would seem to be erroneous. The inability of Thomson to observe a depression of oxidative phosphorylation in spleen mitochondria can be attributed to the fact that his assays were made four hours after irradiation. As can be seen in Table I, the type of effect discussed in this paper can only be observed after 8 hours and cannot be clearly established until 24 hours after exposure to radiation. Although alterations in phosphorylative ability at shorter time periods have been reported (Noyes and Smith, 1959), such effects are generally of very short duration and are not the type discussed by van Bekkum (1957), Benjamin and Yost (1960) or in the body of this paper.

The data presented in this paper agree with previous findings (Jacobson, 1954; Swift, Taketa and Bond, 1954) that radiation-induced damage to the spleen is largely indirect. Both changes in oxidative phosphorylation and histological appearance are produced when radiation is delivered to the upper region of the body alone. Shielding of the head greatly reduces the damage done to the spleen, although the spleen is exposed. These findings are consistent with the suggestion that the directly observable spleen damage is moderated by radiation-induced imbalance (Benjamin and Yost, 1960). This is not to suggest that "direct" effects of radiation do not occur, as is indicated by the data obtained with head-shielded Wistar rats. However, the shielding techniques used in this study permit a low level of radiation to reach the shielded areas, and thus the magnitude of direct effects is difficult to ascertain. Studies of this problem using x-radiation and complete shielding are in process.

Present data do not permit any conclusions about the cause of the radiation damage. The indirect effect of radiation is first detectable by our methods about 8 hours after exposure. The lag between exposure and effect is consistent with the postulate that the primary cause is an induced hormonal imbalance. It has been shown that the pituitary has released thyrotropic hormone one hour after irradiation (Mateyko and Edelmann, 1954) and that the thyroid follicles have been emptied after two hours (Botkin, Praytor, Austing and Jensen, 1952). Histological examination of these glands in this laboratory indicates the same pattern of hormonal release and indicates that such release precedes the observed depression of oxidative phosphorylation by several hours. On the other hand, histological examinations show that a number of cells have already succumbed to the effect of radiation during the first 8 hours after exposure. Consequently, either damage to the phosphorylating mechanism does not cause cell death or the uncoupling of phosphorylation in certain cells precedes that in others. In the latter case a small number of sensitive cells would go undetected by any present assay methods, since the entire cell population from several spleens is used for a single determination. Therefore, if we are to adopt the interpretation that uncoupling of oxidative phosphorylation is a general effect of radiation which is directly or indirectly related to the cause of cell death, it must be assumed that the appearance of a physiological change at 8 hours means that the damage has become *extensive* by that time. Clearly, the data are not sufficient to permit any final conclusion; on the other hand, the indirect effect of the radiation (as seen with shielded spleens) indicates that the hormonal imbalance hypothesis may still be applicable to cell death.

In all cases, recovery of oxidative phosphorylation precedes histological recovery (compare Tables I and II with V and VI). Of course, the data only permit the correlation of the recovery of phosphorylation in the surviving cells of the spleen with the regeneration of certain areas of the spleen whose phosphorylative ability cannot be measured by any present techniques. Since correlations seldom permit conclusions about causality, the data merely indicate the state of the general background against which other processes in the organism are taking place. Although it is attractive to suggest that the recovery of the phosphorylating mechanism is responsible for regenerative processes, it is equally possible that the uncoupling of phosphorylation is a general stress response which permits a more rapid general metabolism required to provide various carbon skeletons for the synthesis of new cellular components. Until it is possible to separate repair processes from recovery of phosphorylation, no critical test of these various hypotheses will be available.

Perhaps the most important point which can be drawn from these data is that protective effects of shielding are protective of the entire recovery mechanism. As Table VII shows, the recovery of liver phosphorylation can be accelerated by shielding of parts other than the liver. Although the acceleration of recovery in the liver is slight, it is nonetheless significant. The standard deviation of the 6-day runs is 0.08 and of the 8-day runs is 0.16. It seems unlikely that in the liver this effect is brought on by a "repopulation of cells from lymphocyte tissue." Rather, the general changes in the organism which occur during the recovery period must have a widespread effect in the various organs of the body.

Although the results presented here are in general agreement with previous work on the inactivation of the phosphorylating mechanism (van Bekkum, 1957; Benjamin and Yost, 1960) and with the increased survival of irradiated rats having part of their body shielded (Jacobson, 1954), it should be noted that the recovery time is much longer than that given previously by Benjamin and Yost (1960). No simple reason can be given for this difference nor for the difference between Wistar and Sprague-Dawley rats. It is probable that the methods previously used to assay P:O ratios were not completely satisfactory, as suggested by Thomson (1964). However, the type of indirect effect observed in these studies depends upon a number of environmental factors and thus can be controlled only with difficulty. A discussion of some of these problems will be presented elsewhere, as it has little bearing on the essential points discussed in this paper.

The authors are indebted to Dr. Hope H. Robson and Ralph E. Barrett for their assistance in the course of the work and the preparation of the manuscript.

SUMMARY

White male rats of the Sprague-Dawley and Wistar strains were exposed to 800 r total-body and partial-body γ-radiation. Estimates of the damage to the oxidative phosphorylation mechanism of spleen, liver and testis of the Sprague-Dawley rats and of the spleen of Wistar rats were made. Similarly, changes in the histology of the spleen follicles were followed, as well as alterations in the circulating lymphocyte population. In general, there seems to be no indication of an initial resistance to radiation; however, there are obvious differences in the recovery rate between the strains and between the total-body and partial-body irradiated animals. Changes in oxidative phosphorylation closely parallel the histological changes and thus can be used as a rapid technique for accessing indirect damage. The data suggest that "radiation resistance" is more a matter of recovery rate than of initial resistance.

LITERATURE CITED

Benjamin, T. L., and H. T. Yost, Jr., 1960. The mechanism of uncoupling of oxidative phosphorylation in rat spleen and liver mitochondria after whole-body irradiation. *Rad. Res.*, **12**: 613–625.

Boche, R. D., and F. W. Bishop, 1950. Studies on the effects of massive doses of X-radiation on mortality in laboratory animals. *In:* Biological Effects of External Radiation, H. A. Blair, Ed., McGraw-Hill Book Co., Inc., New York. Vol. II, Chap. 1.

Botkin, A. L., E. H. Praytor, M. E. Austing and H. Jensen, 1952. Thyroid response to total-body X-irradiation. *Endocrinology*, **50**: 550–554.

Detrick, L. E., H. C. Upham, R. W. Springsteen, R. G. McCandless and T. J. Haley, 1964. Pyridoxine absorption from the small intestine of the X-irradiated rat. *Rad. Res.*, **21**: 186–194.

Glick, D., 1949. Techniques of Histo- and Cytochemistry. Interscience Publishers, Inc., New York.

Hunter, F. E., Jr., 1955. Coupling of phosphorylation with oxidation. *In:* Methods in Enzymology, S. Colowick and W. Kaplan, Eds., Academic Press, New York. Vol. II, sec. III, art. 101.

Jacobson, L. O., 1954. The hematologic effects of ionizing radiation. *In:* Radiation Biology, A. Hollaender, Ed., McGraw-Hill Book Co., Inc., New York. Vol. I, pt. 2, Chap. 16.

Mateyko, G. M., and A. Edelmann, 1954. The effects of localized cathode ray particle irradiation of the hypophysis and whole body X-irradiation on gonadotrophin, thyrotrophin and adrenocorticotrophin of the rat pituitary. *Rad. Res.*, **1**: 470–486.

Metcalf, R. G., R. J. Blandaw and T. B. Barnett, 1950. Pathological changes exhibited by animals exposed to single doses of X-radiation. *In:* Biological Effects of External Radiation, H. A. Blair, Ed., McGraw-Hill Book Co., Inc., New York. Vol. II, Chap. 2.

Noyes, P. P., and R. E. Smith, 1959. Quantitative changes in rat liver mitochondria following whole body irradiation. *Exp. Cell Res.*, **16**: 15–23.

Schneider, W., 1948. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. Biol. Chem.*, **176**: 259–266.

Swift, M. N., S. T. Taketa and V. P. Bond, 1954. Regionally fractionated X-irradiation equivalent in dose to total body exposure. *Rad. Res.*, **1**: 241–252.

Thomson, J. F., 1964. Effects of total-body X-irradiation on phosphate esterification and hydrolysis in mitochondrial preparations of rat spleen. *Rad. Res.*, **21**: 46–60.

Thomson, J. F., and Y. E. Rahman, 1962. Effect of X-irradiation on distribution and properties of cytoplasmic particulates of rat liver. *Rad. Res.*, **17**: 573–578.

van Bekkum, D. W., 1957. The effects of X-rays on phosphorylation *in vivo*. *Biochim. et Biophys. Acta*, **25**: 487–492.

Yost, H. T., Jr., and H. H. Robson, 1959. Studies of the effects of irradiation of cellular particulates. III. The effect of combined radiation treatments on phosphorylation. *Biol. Bull.*, **116**: 498–506.

Vol. 127, No. 2 October, 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

EMBRYOLOGICAL DEVELOPMENT OF THE SYLLID, AUTOLYTUS FASCIATUS (BOSC) (CLASS POLYCHAETA)[1]

M. JEAN ALLEN

Department of Biology, Wilson College, Chambersburg, Pennsylvania, and Institute of Marine Biology, University of Puerto Rico, Mayaguez, Puerto Rico

The Syllidae are unusual among the polychaetes in that some genera of this family reproduce both asexually, by the formation of stolons from a parent stock, and sexually, by the union of gametes from male and female individuals which, in turn, originate from stolons produced asexually. In some instances in this family, some form of gestation accompanies sexual reproduction—the young may develop in a ventral brood sac formed by the female, or eggs and larvae may remain individually attached to the parent (external gestation). A number of papers have been written on reproduction in syllids, and Saint-Joseph (1887) summarized the reproductive methods in the genus *Autolytus* and in the family as a whole. Since then, Potts (1911) has reviewed in considerable detail the various reproductive methods used by the four subfamilies of syllids, and recently Pettibone (1963) has given a brief summary of their reproductive methods. However, surprisingly little has been published on the embryology of this very interesting family of polychaetes, and most of this was written 50 to 120 years ago. Cleavage has been described and pictured in only a few papers on syllid reproduction (for example, Malaquin, 1893, the most extensive paper on syllids; Viguier, 1884—only the first three cleavages are shown; Pierantoni, 1903; and Schneider, 1914). Descriptions and figures of syllid larvae are scattered throughout the literature (Oersted, 1845; Müller, 1855; Agassiz, 1863; Pagenstecher, 1863; Greeff, 1879; Viguier, 1884; Saint-Joseph, 1887; Pierantoni, 1903; Herpin, 1926; Okada, 1930; Dales, 1951; *et al.*). However, descriptions depicting a series of developmental stages from early cleavage through several larval stages for any one species of syllid are rare. The writer has noted only two such descriptions in the literature (Viguier, 1884, and Pierantoni, 1903) and these belong to a different subfamily (Exogoninae) than does *Autolytus* (Subfamily Autolytinae). All figures in these two papers appear to be drawings of external views, and in Viguier's paper, the series on *Exogone gemmifera* does not include the stages between 8-cells and gastrulation.

---

[1] This research was supported by grants G-8775 and G-19958 from the National Science Foundation.

Copyright © 1964, by the Marine Biological Laboratory

The writer has found embryological stages of *Autolytus fasciatus* abundant in Puerto Rican waters (Allen, 1957a, as *A. ornatus;* see Pettibone, 1963, for synonymy) and has reported observations on their histochemistry (Allen, 1957b, 1961). Since there appears to be no description of the embryological development of *A. fasciatus* in the literature, it seems advisable to publish the material now available on the normal embryology of this species, both to serve as background for continued histochemical studies and as an addition to our rather sparse knowledge of the development of this interesting group of polychaetes.

The genus *Autolytus* shows a striking sexual dimorphism (first clearly demonstrated by Agassiz, 1863, for *A. cornutus*) in the individuals that produce the gametes, the female often being referred to as the sacconereis form and the male as the polybostrichus. According to Potts (1911), gestation occurs in all forms of the subfamily Autolytinae. In the case of *Autolytus fasciatus,* the female, or sacconereis, forms a ventral sac which encloses the embryological stages from the fertilized egg through various differentiating larval stages (Fig. 25). This sac is often referred to as an egg sac although the term "brood sac" is perhaps a more accurate and more descriptive term. The present paper deals with the development of embryological stages within the ventral brood sac of the red-banded *Autolytus, A. fasciatus.*

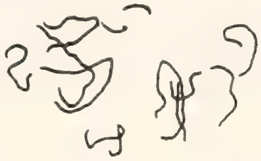
Materals and Methods

Procuring and handling living developmental stages. As noted earlier (Allen, 1957a), night collections of the sacconereis or female can be made at almost any time of the year at the laboratory dock on the island of Magüeyes, located off La Parguera, Puerto Rico. Collections were made by use of a reflector bulb and a dip net. Individuals rise to the surface within the circle of light from a depth of several feet. When abundant, as many as 50 females (measuring approximately 13 mm. in length) have been collected with one dip of the net, and as many as 165 have been collected within an hour. During the year 1955, the females with ventral brood sacs were found to be most abundant during the last half of May and June, the middle of July, and the first part of September (Allen, 1957a); again in May and in early September of 1963 they were found to be abundant.

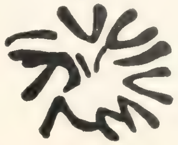
The brood sac of this species often gives the impression of having two, or more often three, lobes in linear arrangement. The ventral sac of any one female was found to contain just one developmental stage (Fig. 25); individuals could be seen through the thin wall of the brood sac. Some indication of the stage of development can be obtained from the color of the stage (as seen *en masse*) within the brood sac. There are some variations but if the brood sac appeared white and small, it generally contained early to mid-cleavage stages, while if it appeared blue or deep lavender, it usually contained rather smooth-surfaced spherical stages (late cleavage to gastrulae, or sometimes elongating trochophores). The brood sacs tend to appear lighter again as the trochophores develop and elongate, and then become a creamy-yellow color as swimming larvae develop. During these later larval stages, observations made through a dissecting microscope reveal red dots which are the eyes of the developing larvae, and they, together with the white color of the larval bodies, give an overall yellowish color to the brood sac.

For photomicrographs and for a more detailed study of the living organisms, developing stages were freed from the brood sacs by using a #5 watchmaker's forceps and a dissecting microscope. Isolated individuals could then be viewed under low power of a compound microscope. Some of the earlier stages were isolated in this way in small stender dishes and were allowed to develop for a period in order to obtain an estimate of the time it took to develop from one stage to another. For such studies, and for photomicrographs, the ciliated stages were slowed down with a little dry MS-222 (tricain) added with a dissecting needle to a drop of filtered sea water containing the larvae (optimal concentrations for quieting various larval stages were not determined).

Handling of fixed material. Isolated stages were sometimes fixed for sectioning but, in most cases, once the stage had been determined by slitting the brood sac, the whole female with her brood sac of developing stages was fixed.



1



2

FIGURE 1. A camera lucida drawing of a prophase from an early trochophore (squash preparation, alcoholic HCl-carmine). Note the six pairs of chromosomes of the diploid set.

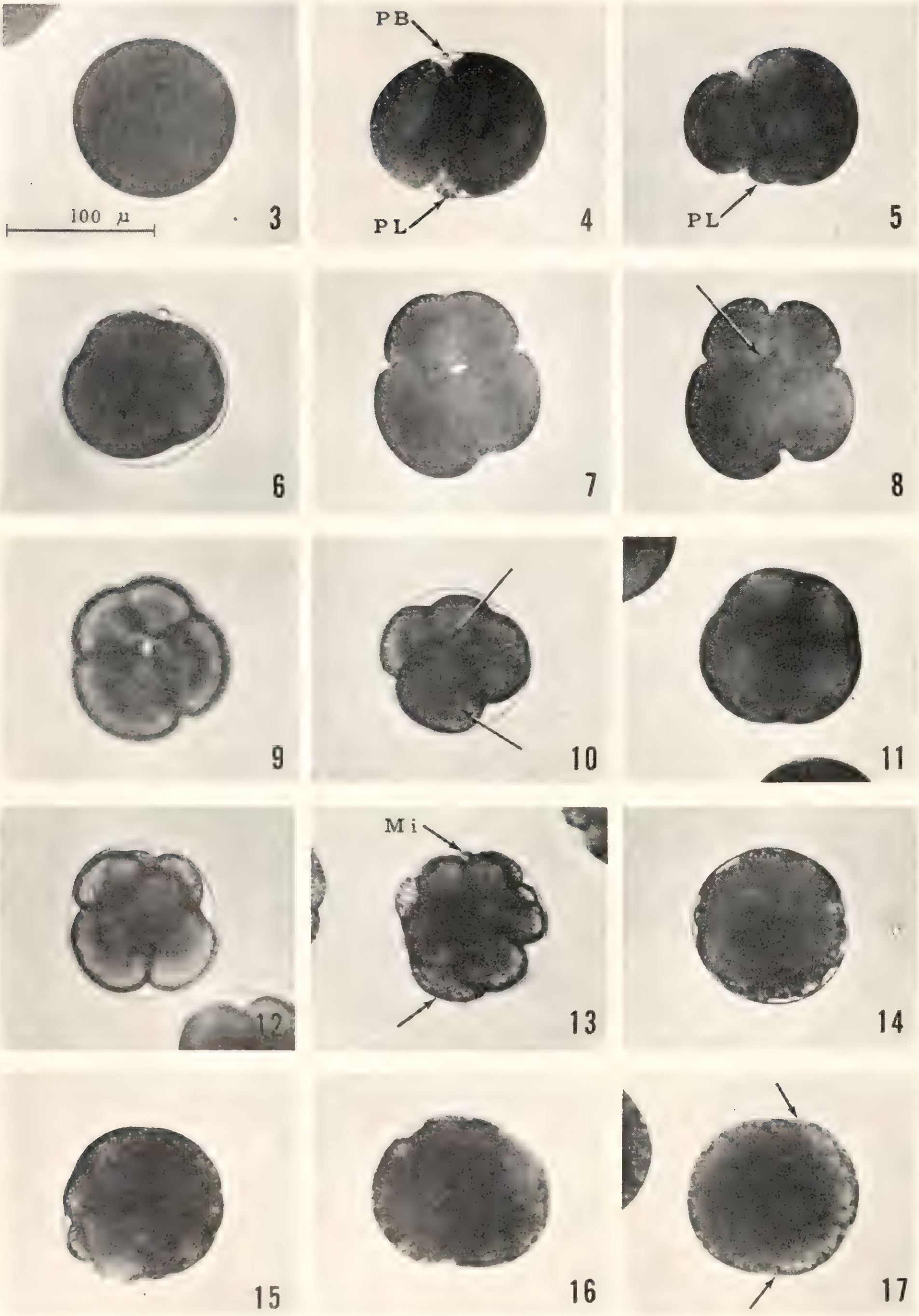
FIGURE 2. A camera lucida drawing of a polar view of a metaphase from an early trochophore (squash preparation, alcoholic HCl-carmine). Note again the 12 chromosomes of the diploid set.

The main fixatives used were acetic-alcohol (1:3), 80% ethyl or isopropyl alcohol, formal-calcium, picro-alcohol-formalin (Rossman's fixative), and Schaudinn's fixative. Most of the stages (usually while still in the brood sac attached to the female) were embedded in paraffin and sectioned serially at 5 to 8 micra; some, however, were stained as whole mounts. A number of stains were used including Harris' hematoxylin with or without eosin, azure B, gallocyanin chromalum, Pollak's trichrome, and fast green with Feulgen. Pollak's trichrome was particularly useful in determining the extent of the ciliated bands.

There seems to be no record in the literature for the chromosome number of any species of *Autolytus,* so in order to determine the diploid chromosome number for *A. fasciatus,* squash preparations of cleavage to early larval stages were made. Attempts at making preparations by the ordinary aceto-carmine technique for chromosomes were unsuccessful, but immediate success was obtained with the alcoholic HCl-carmine technique of Snow (1963). Photomicrographs and camera lucida drawings were made of the chromosomes.

DESCRIPTION OF DEVELOPMENT

Time table of development. It is difficult to construct a time table of development for *A. fasciatus* because the stages normally develop within the ventral brood


PB
100 μ
3
PL
4
PL
5
6
7
8
9
10
11
12
Mi
13
14
15
16
17

FIGURES 3–17.

sac attached to the female and the time of fertilization is unknown. However, an approximation was reached by two methods: (1) by liberating a few developing organisms from the sac and noting the stage of development, and then noting the time it took them to reach a later stage; (2) by carefully liberating a few embryos from the brood sac of a single female at various intervals and noting the progress of development. In the rare cases when undivided eggs were observed, it took them 30 minutes to two hours to cleave. Using this as a basis, the times given are approximations at temperatures of 25.5–29° C.

Stage	Time
Two-cells	2 hours
Four-cells	3½–4½ hours
Eight-cells	5–6 hours
Mid-cleavage	7–9 hours
Late cleavage	10–14 hours
Pre-trochophores	24–28 hours
Trochophores	1½–2 days
Post-trochophores	2½–3 days
Unhatched swimming larvae and hatching larvae	3½–6½ days

Chromosome number. Favorable views of squash preparations demonstrate that the diploid chromosome number for *A. fasciatus* is 12. The 12 chromosomes

---

Figures 3 through 23, except for Figure 20, are photomicrographs of living stages, all taken at the same magnification. The scale indicating magnification is shown on Figure 3 and is repeated on Figure 18. Figure 20 is a photomicrograph taken from a squash preparation. Figures 24 through 45 are photomicrographs of sectioned material. Figure 24 and Figures 28 through 43, except for Figures 36, 40, and 42, were all taken at the same magnification; the scale is indicated on Figure 28, and is repeated on Figures 30 and 38.

FIGURE 3. Unfertilized egg, with numerous yolk spheres making it so opaque that the germinal vesicle is not visible.

FIGURE 4. Two-cell stage, showing that the AB blastomere is somewhat smaller than the CD. Note the polar lobe (PL) and one of the polar bodies (PB).

FIGURE 5. Two-cell stage, showing the polar lobe (PL) being withdrawn into the CD blastomere.

FIGURE 6. Second cleavage furrow beginning. Note the two polar bodies held within the fertilization membrane.

FIGURE 7. Central hole appearing as the second cleavage furrow becomes evident.

FIGURE 8. Polar view showing a tongue-like extension of the D-blastomere. This extension (arrow) may represent a second polar lobe.

FIGURE 9. Four-cell stage showing distinct cleavage furrows and persistence of central hole.

FIGURE 10. Four-cell stage completed with the obliteration of the hole as the blastomeres move over one another to form the cross-furrow (at tip of upper arrow). Note that the D-blastomere (at lower arrow) is larger than the others.

FIGURE 11. First and second cleavage furrows becoming indistinct just prior to initiation of the third cleavage furrow.

FIGURE 12. Lateral view of eight-cell stage, showing two tiers with four slightly smaller micromeres towards the animal pole.

FIGURE 13. Early to mid-cleavage showing one of the smaller micromeres (Mi) and the largest macromere (lower arrow), probably the D-blastomere.

FIGURES 14 and 15. Late cleavage stages, showing closely packed cells, and outer edge becoming smoother as surface blastomeres become smaller.

FIGURE 16. Probably a normal gastrula stage although all batches of eggs do not show this irregular shape.

FIGURE 17. Very early trochophore (anterior at right). Arrows indicate where cilia of prototroch will penetrate the larval membrane.

may be counted in the prophase stage shown in Figures 1 (a camera lucida drawing) and 20 (a photomicrograph of the same prophase). A polar view of the metaphase in which all the chromosomes may be counted is shown in Figure 2 (a camera lucida drawing). These three figures were made from squash preparations of early trochophores. So far as the writer has determined from the literature, the chromosome number of no other species of *Autolytus* has been determined.

Unfertilized eggs. Before being released into the brood sac, white spherical eggs, measuring approximately 100 micra in diameter, are packed within the coelom of the female along the entire length of the body. Hartman (1945) describes the eggs of this species as bright blue but she must have been referring to later spherical stages as described above; this undoubtedly also accounts for Pettibone's reference (1963, page 142) to these eggs as "whitish, bright blue or purplish." No polar bodies were observed in these coelomic eggs, and sectioned material reveals that most of the oöcytes are in metaphase I (Fig. 28). Presumably the oöcytes remain in this phase until fertilization occurs, for polar bodies have not been noted until after fertilization when they were observed in sectioned material of fertilized eggs within the brood sac (Figs. 24 and 29; note spermatozoan heads in Figs. 24 and 27). A photomicrograph of a living egg is shown in Figure 3. Viewed with the light microscope, the cytoplasm of the egg in sectioned material appears as a network of fine granules surrounding the closely packed yolk spheres (compare Figs. 26, 28, and 30—yolk spheres are stained only in Fig. 30). The cytoplasmic granules are more concentrated peripherally, forming a thin cortical layer (compare Figs. 26 and 28). This cortical layer is bounded by a thin transparent vitelline membrane which is difficult to see except where it has been pulled away from the egg surface (Fig. 26).

Fertilization. Fertilization was not observed in the present investigation, the male form having been seen only rarely, and usually not at the dock where females were collected but over a nearby reef. Spermatozoa, however, were observed in sectioned material within egg sacs containing undivided eggs or early cleavage stages. These spermatozoa may be seen associated with the eggs (Fig. 24) or stuck to the inside of the egg sac (Fig. 27—the two sac-like protrusions on the midpiece of the lower spermatozoön may be an abnormality).

The writer has found only a few references to fertilization in syllids and no exact description of the process. Fertilization in most instances appears to be external, occurring either after swarming and release of gametes into the sea or, in cases of external gestation, after the eggs have been released and attached externally to the female (Viguier, 1884; Pierantoni, 1903; Galloway and Welch, 1911; Herpin, 1923, 1924a, 1924b, 1926; *et al.*).

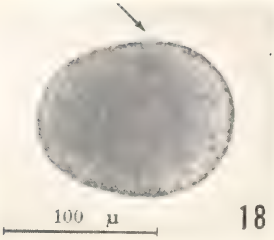
In the Subfamily Autolytinae, Herpin (1926) describes the ventral brood sac as being formed by the female at the moment of egg laying. L. Dehorne (1918, from Gravier, 1923) described and pictured the formation of the egg sac in *Myrianida;* as the egg sac is secreted, the eggs pass through the modified nephridia of the female into this ventral sac. They thus must become fertilized before the egg sac is completed. According to Gidholm (personal communication; and 1964, Zool. Bidr. Uppsala, in press), fertilization in *Autolytus edwarsi* is external; the sexual forms swarm at the surface and the male swims rapidly around the female ejecting "a mucous sperm which forms a belt around the female." The eggs are not exuded immediately and the brood sac is formed by mucous glands in the bases of the parapodia; as the eggs exude from genital ducts (modified nephridial ducts), they are fertilized immediately. The eggs are shed in metaphase I and upon sperm entrance form two polar bodies, thus completing the meiotic divisions. Gidholm has observed mating behavior in the laboratory in several other species of *Autolytus* besides *A. edwarsi*, so he feels that the above description is probably typical of the genus. It seems likely, then, that this description of fertilization would apply to *A. fasciatus*.

As in a number of other annelids, no raised fertilization membrane is produced in *A. fasciatus* (Figs. 4, 7, and 8). The polar bodies as a consequence appear to lie for a time in a depression on the egg surface (Fig. 29). Sections of a small clump of uncleaved eggs revealed the male and female pronuclei prior to the formation of the first cleavage spindle (Fig. 30).

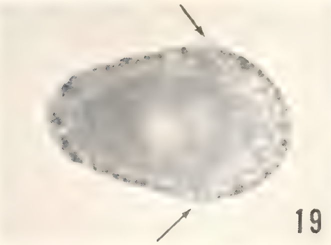
Cleavage. Females with uncleaved eggs in the brood sac, or with two-cell stages, were extremely rare in the collections made. However, in the spring of 1963, several such females were observed and were used to obtain further information regarding early cleavage. A series of photomicrographs was made as these living eggs cleaved. Cleavage is total, unequal, and spiral. The blastomeres are held closely together by the vitelline membrane (obvious only at the cleavage furrows) so that they are somewhat flattened against one another. This flattening of the blastomeres is not very marked in some of the photomicrographs of living stages (Plate I); in these stages it is probable that the heat of the lamp caused the egg membrane to swell slightly so that the blastomeres rounded up more than is typical. This, however, had the advantage of making the early cleavage pattern and spatial relationships of the blastomeres easier to determine. Eggs and blastomeres are so opaque that chromosomes and spindles were not observed in living material.

The first cleavage furrow is meridional, resulting in two blastomeres of unequal size, the AB being somewhat smaller (approximately 90 × 50 micra) than the CD blastomere (approximately 100 × 70 micra, Fig. 4). At this two-cell stage a polar lobe may be observed at the vegetal pole; it is subsequently gradually withdrawn into the CD blastomere (compare Figs. 4 and 5). As the second cleavage furrow forms, a hole appears between the cleaving blastomeres (Fig. 7), and then a tongue-like extension of the D-blastomere was sometimes observed in the region where the cross-furrow will form (Fig. 8). Although material was scarce, the few cases observed suggest that the tongue-like extension may represent the formation of another polar lobe as seen from a vegetal pole view (compare Fig. 8 with Wilson, 1904, Fig. III, 15 for *Dentalium*).

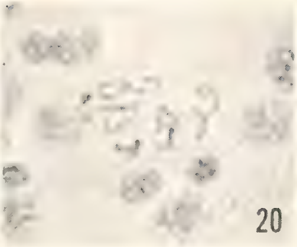
During the first and second cleavages in some molluscs (for example, *Ilyanassa* and *Dentalium*) strikingly large polar lobes are formed (Raven, 1958, Fig. 19 after Morgan; and Wilson, 1904, Figs. I and II). The formation of polar lobes seems to be relatively rare in polychaetes. In the case of *Autolytus* described here, the polar lobes are more nearly the size of the relatively small ones formed in the early cleavage of the polychaete, *Chaetopterus* (compare Figs. 4 and 5 with Mead, 1897, Plate XIX, Figs. 118 and 119). The only reference noted in the literature describing the formation of polar lobes in the cleavage of syllids was that of Schneider (1914) in which he diagrammed the first polar lobe (Fig.



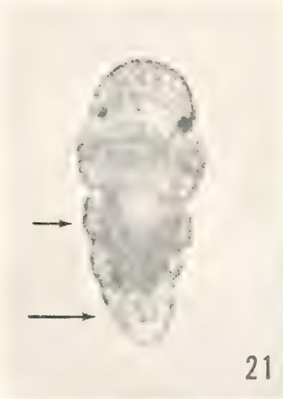
18



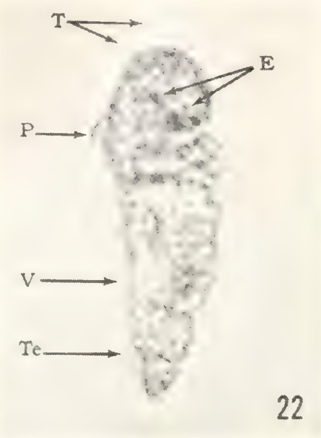
19



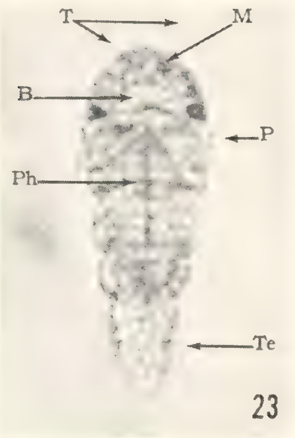
20



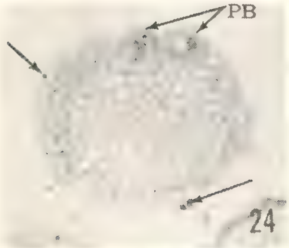
21



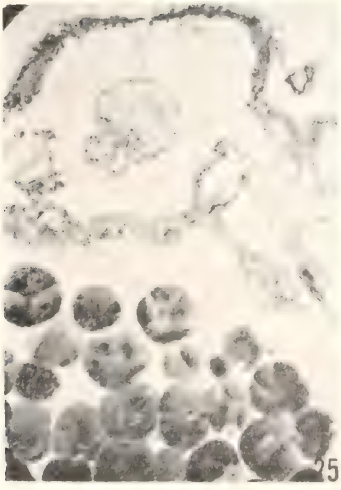
22



23

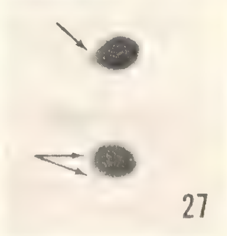


24

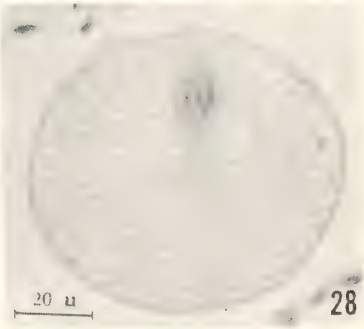


25

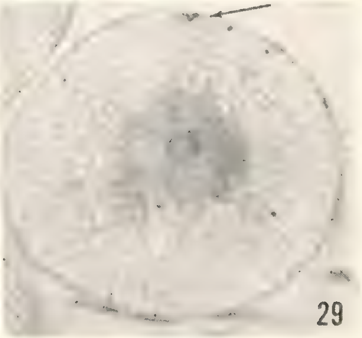
26



27



28



29

FIGURES 18–29.

4, page 625) and referred to the formation of a second (page 625). This short paper by Schneider is concerned with the early development of *Pionsyllis pulligera* which belongs to the subfamily Eusyllinae, a different subfamily from that to which *Autolytus* belongs (Subfamily Autolytinae).

The hole which may be observed as the second cleavage furrow forms (Fig. 7) remains until the furrows of the four-cell stage become distinct (Fig. 9) but is subsequently obliterated as the blastomeres move over one another to form the cross-furrow (Figs. 10 and 31) which is typical of spiral cleavage. The resulting C and D blastomeres are larger than the A and B cells, the D blastomere being the largest (Fig. 10).

Foreshadowing the initiation of the third cleavage furrow, the two furrows of the four-cell stage become indistinct (Fig. 11). The third cleavage furrow is horizontal; its completion results in a tier of four somewhat smaller micromeres lying over four macromeres (Fig. 12; compare with Malaquin, 1893, Plate XIV, Figs. 4 to 10, in which the size discrepancy between early micromeres and macromeres is much greater in the closely related genus, *Myrianida,* than in *Autolytus*). The large blastomere which persists as cleavage continues is probably the D macromere (Fig. 13) and presumably gives rise to the mesoderm of the larva. Later cleavages are asynchronous (Figs. 13 to 15, and 32); the

Figure 18. Dorsal view of an early trochophore without eyes (anterior to right). Arrow indicates beating cilia of the prototroch which have penetrated the larval membrane.

Figure 19. Dorsal view of an older trochophore showing prototroch (arrows), two eyespots just anterior to the prototroch, and darker central yolk mass with a pharynx differentiating at the level of the prototroch.

Figure 20. Squash preparation from an early trochophore stage showing a prophase with the diploid set of twelve chromosomes. Alcoholic HCl-carmine.

Figure 21. Dorsal view of an elongated post-trochophore with two prominent eyespots, three ciliated areas (prototroch is clear; arrows indicate level of more posterior cilia), and differentiating central gut. Anterior tactile bristles are present but are not clearly shown.

Figure 22. Lateral view of late unhatched swimming larva, showing tactile bristles (T), two of the four eyes (E), prototroch (P), telotroch (Te), and beating ventral cilia shown as hazy line (V).

Figure 23. Dorsal view of a partially contracted larva similar to that in Figure 22, showing anterior tactile bristles (T), anterior mucous cells (M), brain (B) between the two larger eyes, prototroch (P), pharynx (Ph), and telotroch (Te). The middle cilia are not visible.

Figure 24. Section through a fertilized egg near the animal pole, showing the two polar bodies (PB), and two sperm heads (visible at arrows—the larger dark spot at lower arrow is not a sperm). Gallocyanin.

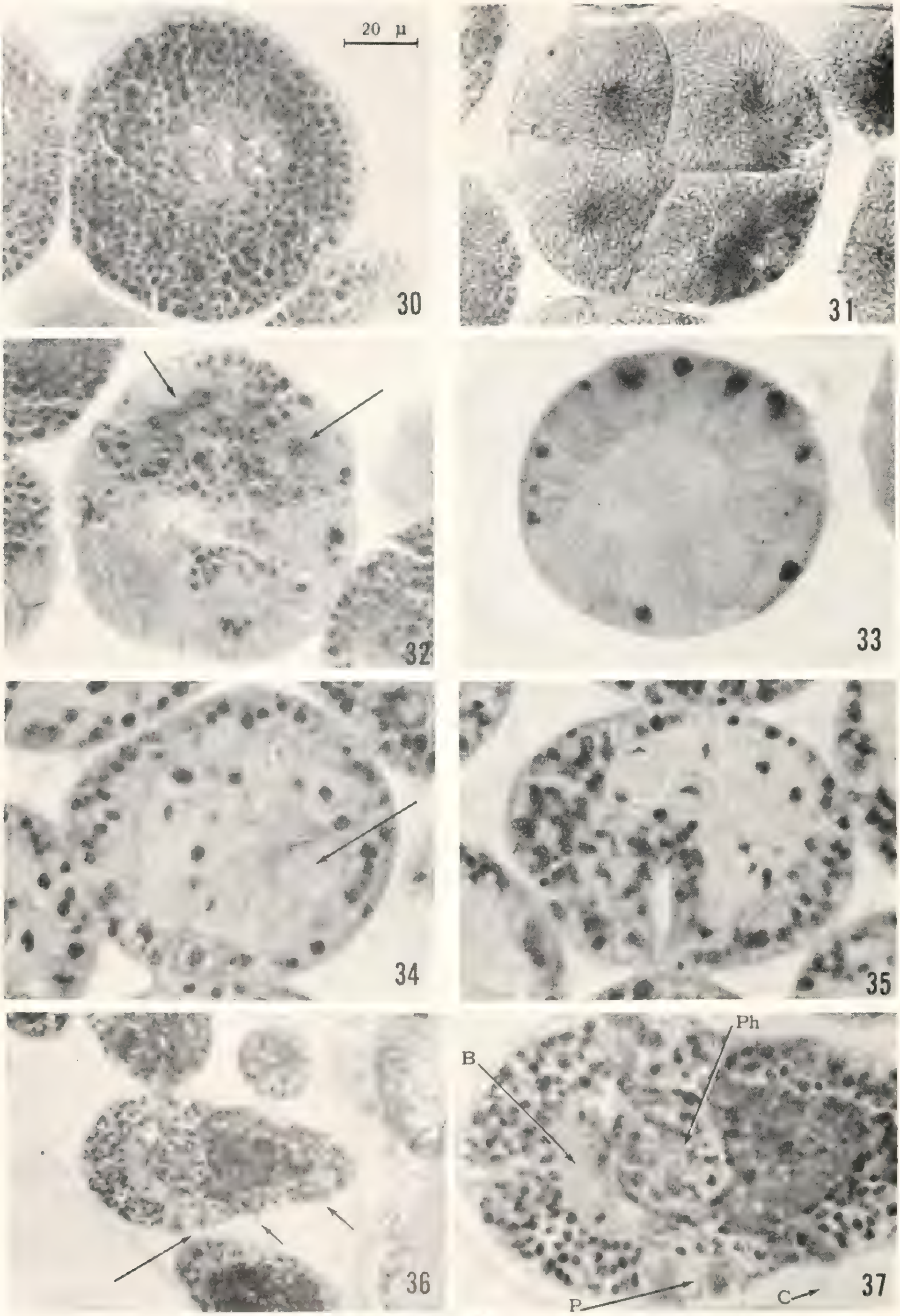
Figure 25. Transverse section through a female, or sacconereis, showing a portion of the ventral brood sac packed with early cleavage stages. Pollak's trichrome.

Figure 26. Enlargement of a portion of a sectioned egg, showing a network of fine granules surrounding the closely packed yolk spheres (yolk is unstained) and a cortical concentration of granules. Note the thin vitelline membrane where it has been pulled away from the surface (at arrow). Gallocyanin.

Figure 27. Two spermatozoa caught on the inner mucous surface of the brood sac and seen in sectioned material. The flagellum of the upper sperm was barely visible (arrow indicates lighter midpiece). The two sac-like protrusions on the midpiece (arrows) of the lower sperm may be an abnormality. Pollak's trichrome.

Figure 28. Primary oöcyte in metaphase I shown inside the coelom of the sacconereis (yolk is unstained). Gallocyanin.

Figure 29. A fertilized egg showing chromosomes in first cleavage. Note the two polar bodies (at arrow) lying in a depression on the egg surface, and the absence of a raised fertilization membrane. Gallocyanin.


20 μ
30
31
32
33
34
35
36
Ph
B
P
C
37

FIGURES 30–37.

opaqueness of the blastomeres, due to their stored yolk, makes it very difficult to work out the details. The apical rosette and annelid cross, characteristic of polychaete development, have occasionally been observed in living material. Late cleavage to blastula stages appear in living material as spheres of closely packed small cells with the outer edge becoming smoother as surface blastomeres become smaller (compare Figs. 14 and 15).

Gastrulation. Both living stages and sectioned material indicate that gastrulation is accomplished by epiboly, the micromeres growing over the endodermal macromeres (compare Figs. 33 to 35), much as in the case of *Sphaerosyllis* (Pierantoni, 1903, Plate II, Figs. 19 and 20) and *Myrianida* (Malaquin, 1893, Plate XIV, Figs. 7 to 10). The gastrula of *Autolytus fasciatus* may appear in the living state as somewhat irregular in shape (Fig. 16). The subsequent larval stages are described below.

Trochophores. Young trochophores are ovoid but are becoming broader anteriorly where the prototroch is differentiating (compare Figs. 17 and 18). In living stages the prototroch is first observed as a thickened area around the broadest part of the anterior end. In the larva shown in Figure 17, the cilia of the prototroch have not yet penetrated the larval membrane. As individual cilia penetrate the membrane (Fig. 18), they begin to beat, and soon the trochophore is moving about in place. A telotroch also is beginning to differentiate but is so finely ciliated at this stage that it is difficult to observe in younger members of this stage, such as the larva in Figure 18. *A. fasciatus* appears to be precocious in the development of these ciliated bands, compared with *A. edwarsi* (Malaquin, 1893, Plate XIV). The younger stages have not yet developed eyes and the older ones have two orange-red eyespots just anterior to the prototroch (compare Figs. 18 and 19). Centrally, just behind the prototroch, the larval pharynx is differentiating; in sectioned material, the stomodeal invagination is clearly seen (Fig. 35). The post-pharyngeal gut is obvious in living and sectioned material as a dense central mass of endodermal cells, tapering posteriorly and packed with yolk spheres (Figs. 19 and 35). The cilia of this and later stages appear to penetrate the original egg membrane which has thus become the larval cuticle; as noted earlier (Allen, 1959, *et al.*) this is not un-

FIGURE 30. Fertilized egg, showing male and female pronuclei near center. Yolk spheres are stained. Pollak's trichrome.

FIGURE 31. Four-cell stage, showing cross-furrow in center. Pollak's trichrome.

FIGURE 32. Early cleavage stage. Note anaphase at upper left (arrow) and polar view of metaphase at upper right (arrow). Pollak's trichrome.

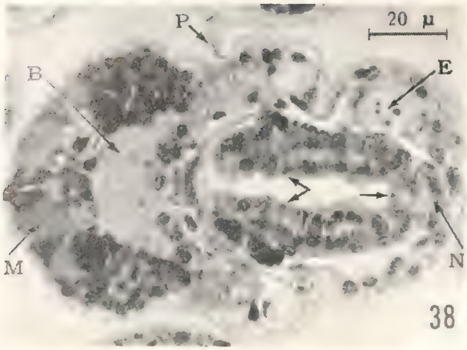
FIGURE 33. Later cleavage stage. Note epibolizing micromeres, and central yolk mass. Harris' hematoxylin and eosin.

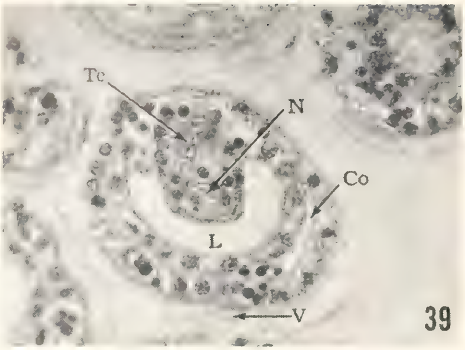
FIGURE 34. Stage just prior to stomodeal invagination. Note anaphase in an endodermal cell (arrow). Harris' hematoxylin and eosin.

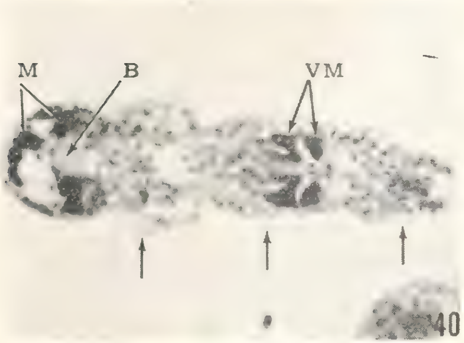
FIGURE 35. Sagittal section through an early trochophore without eyes (anterior at left), showing stomodeal invagination. Note central mass of yolky endodermal cells. Harris' hematoxylin and eosin.

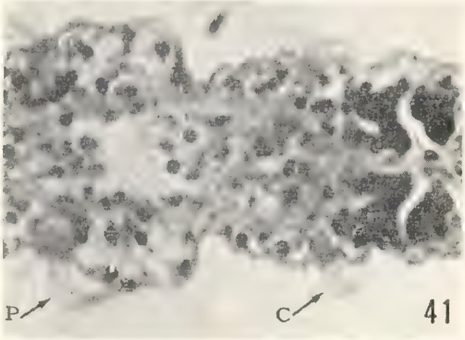
FIGURE 36. Frontal section through a post-trochophore (anterior at left), showing three ciliated areas (arrows), brain, pharynx, and undifferentiated post-pharyngeal gut showing as a dark central yolk mass. Section does not include mucous cells. Feulgen and fast green.

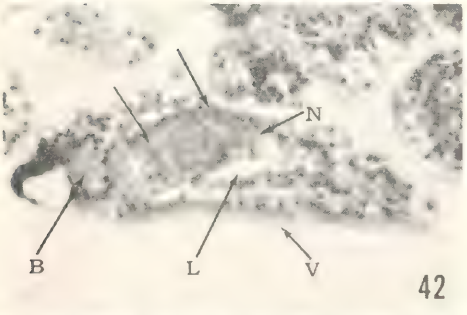
FIGURE 37. Enlargement of anterior two-thirds of post-trochophore shown in Figure 36. Note differentiating brain (B), pharynx (Ph), large prototrochal cells (P), and lateral ciliated tuft (C).

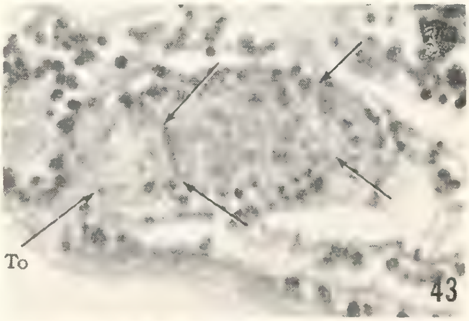

P
20 μ
B
E
N
M
38

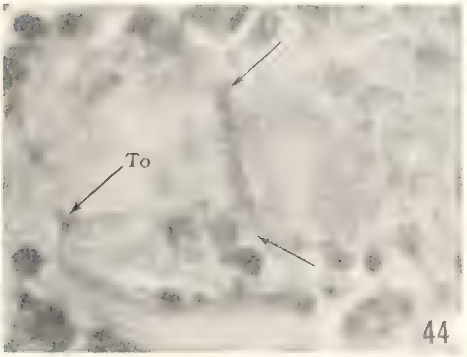


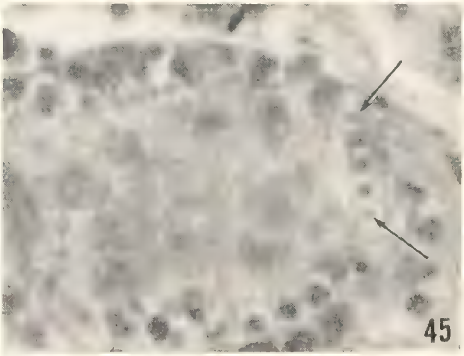












FIGURES 38–45.

common among polychaetes. Several investigators (Barrois, 1877; Viguier, 1884; Malaquin, 1893; Thorson, 1946) have noted this origin of the larval cuticle in connection with the development of syllids. Pierantoni (1903), however, does not agree with the interpretation that the larval cuticle represents the original egg membrane.

Post-trochophores. This stage has elongated, and a pair of lateral ciliated tufts has developed between the active prototroch (a complete band of long cilia) and telotroch (Figs. 21, 36, and 37). The ventral surface has also developed cilia. Similar ventral cilia have been pictured for *A. cornutus* (Dales, 1951, as *A. prolifer*—see Pettibone, 1954) but not for *A. edwarsi* (Malaquin, 1893). The larvae move slowly about on the bottom and the more active are able to swim to the surface, rotating about their longitudinal axes. Younger members of this group still have only two eyespots, but the older larvae are developing a second pair of eyes, and also have at least two slender spine-like structures extending from the anterior surface (not clear in Fig. 21).

In addition to the above, sectioned material reveals that a number of large cells, probably mucous (Allen, 1961), have differentiated at the anterior tip of the head. The brain is differentiating, and the pharynx and post-pharyngeal gut are clearly defined although the latter is still relatively undifferentiated (Figs. 36 and 37).

Late unhatched swimming larvae and newly hatched larvae. The brood sacs containing these unhatched larvae are usually large and appear pale yellow in color. The late unhatched and newly hatched larvae are essentially similar, but the hatched ones are very active swimmers and are positively phototactic, collecting at the edge of the dish nearest the light, thus making it easy to remove them for fixation or for changing the sea water. These swimming larvae measure approximately 300 × 100 micra; the ventral surface is covered by cilia (Figs. 22, 39,

---

FIGURE 38. Frontal section through anterior region of a late unhatched swimming larva, showing large mucous cells (M) and brain (B) in the cephalic segment, and pharynx (with pharyngeal teeth indicated by central arrows). Note also the prototroch (P), the large endodermal sphere (E, containing yolk granules) within the wall of the intestine, and the narrow post-pharyngeal area of the gut (N) shown in transverse section.

FIGURE 39. Transverse section through a larva similar to that in Figure 38, showing the posterior end of the pharynx with two lateral plates of pharyngeal teeth (Te), the narrow area of the gut (N) pushing into the lumen (L) of the anterior part of the intestine. Note also the coelom (Co) between intestine and body wall, and the ventral cilia (V).

FIGURE 40. Frontal section through a larva similar to that in Figures 38 and 39, showing anterior mucous cells (M), brain (B), and group of prominent ventral mucous cells (VM). Note also the levels of prototroch, lateral ciliated tuft, and telotroch (arrows).

FIGURE 41. Enlargement of central portion of the larva shown in Figure 40, with prototroch (P), lateral ciliated tuft (C), and ventral mucous cells.

FIGURE 42. Sagittal section through a larva similar to those in Figures 38 to 41, showing the brain (B), and pharynx with anterior and posterior pharyngeal teeth (level of arrows). Note also the narrow section of the gut (N), and lumen (L) of anterior part of intestine. The hazy layer on the lower surface represents the ventral cilia (V).

FIGURE 43. Enlargement of pharynx shown in Figure 42. Note single large tooth (To) near the mouth, the double row of anterior teeth (between anterior pair of arrows), and row of five posterior teeth (between posterior arrows).

FIGURE 44. Enlargement of single anterior tooth (To), and double row of anterior teeth (between pair of arrows) shown in Figure 43.

FIGURE 45. Enlargement of the five posterior teeth (between arrows) shown in Figure 43.

42, and 43). The larvae have a segmented appearance but the segments are not yet completely demarcated. These early larval segments are described in the following paragraphs. Most of the structures described can be observed in living material.

(1) *Cephalic segment.* The cephalic segment bears two pairs of red eyes, one pair being smaller and located slightly posterior, median, and dorsal to the larger pair; each of the larger pair has developed a lens. The differentiating brain can be observed in living larvae as a whitish area between the two larger eyes (Fig. 23) and is clearly seen in sections (Figs. 38, 40, and 42). Anterior to the brain several large mucous cells are arranged symmetrically in a curved row following the external contour of the head (Figs. 23, 38, and 40). In favorable preparations of living larvae, both nucleus and nucleolus may be observed in these cells. Several anterior slender spine-like structures are visible on the head (Figs. 22 and 23). Similar structures are shown in the drawings of larvae of several other species of syllids (Greeff, 1879; Malaquin, 1893; Herpin, 1926; Okada, 1930; Dales, 1951). Some investigators refer to them as tactile hairs or bristles (Herpin, 1926, described them as "poils tactiles" or "cils tactiles raides"; Greeff, 1879, uses the term "Tastborsten" or "Hautstacheln"). In favorable preparations of living specimens, close examination of these tactile bristles under a magnification of × 430 revealed that they are actually very slender tufts of fine cilia. Thus, structurally they suggest a slender version of the cirri characteristic of certain protozoon ciliates (order Hypotricha). Two minute short anterior tufts of cilia may also be observed just median to these anterior tactile bristles, and also a minute median tactile bristle can be seen between these two small tufts. These latter cilia are so small and delicate they do not show up in the photographs. Short, fine cilia can be seen just anterior to the prototroch; they are difficult to observe clearly but appear to arise from the posterior edge of the cephalic segment.

(2) *Anterior pharyngeal segment* and (3) *Posterior pharyngeal segment.* The band of short cilia described in the preceding paragraph appears to be almost confluent with the prototroch, which is the prominent band of longer, very active cilia borne on the anterior part of the anterior pharyngeal segment (Figs. 23, 38, and 41). Just posterior to the prototroch is a pair of fragile-looking spine-like structures which are probably similar in structure to the anterior tactile bristles; similar lateral tactile bristles have been pictured in other syllid larvae of the subfamilies Autolytinae and Eusyllinae (Malaquin, 1893; Herpin, 1926; Okada, 1930).

Internally, at the level of the prototroch, the anterior edge of the pharynx is visible (Figs. 23 and 38). The pharynx extends through the rest of this segment and through the anterior part of the posterior pharyngeal segment (Figs 23 and 38), where it narrows to form a short section (Figs 38, 39, and 42). This short narrow portion, in turn, joins the differentiating intestine which has developed a relatively large lumen at this level (Figs. 39 and 42).

The spatial relationships of the larval pharynx, the anterior end of the intestine, and the narrow portion of the digestive tract in between, arise in the following way. During its formation, the stomodeal invagination pushes into the anterior gut endoderm. Subsequently, the larval pharynx differentiates from the invaginated ectoderm, and the intestine differentiates from the yolk-filled endoderm. In the meantime, the narrow portion—if its origin is similar to that described for other larval syllids (Malaquin, 1893, *et al.*)—arises from a posterior proliferation of the larval pharynx. As a consequence of these processes, the anterior end of the intestine in these swimming larvae surrounds the posterior end of the larval pharynx (Figs. 38, 39, and 42), and the narrow post-pharyngeal gut appears to push into the anterior portion of the differentiating intestine (Figs. 38 and 39). This narrow section between the larval pharynx and the differentiating intestine may represent the primordium of the posterior part of the adult pharynx, together with the proventriculus and ventriculus, as the subsequent development of this area has been described for other syllids (Malaquin, 1893; Pierantoni, 1903; Herpin, 1926), including *Autolytus edwarsi* (Malaquin, 1893). It will be necessary, however, to study later larval stages before this can be confirmed for *Autolytus fasciatus.*

Large spheres seen at the level of the pharynx superficially suggest the precocious development of eggs. However, they are associated with the lining of the intestine (Fig. 38); position and staining reactions suggest them to be the remains of some of the yolk-filled endodermal cells of the post-trochophore stage rather than developing eggs.

The posterior pharyngeal segment bears a pair of prominent ciliated tufts (Figs. 40 and 41) described above as appearing in the post-trochophore stage. Just anterior to these lateral tufts of cilia is a second pair of tactile bristles (similarly placed bristles are shown for *A. pictus* in Okada, 1930, Fig. 2A). Internally, pharyngeal teeth are differentiating. At least two relatively large teeth may be seen at the anterior end of the pharynx (one of these is shown in Figs. 43 and 44). About one-third the length of the pharynx, a circle of teeth has differentiated (one lateral set of these teeth is shown in Figs. 43 and 44). Near the posterior end of the pharynx, there is a set of somewhat stouter teeth arranged in two lateral halves (compare Figs. 43 and 45 with 39). Six teeth have been counted in each half of this posterior set (five are visible in Fig. 45). The narrow post-pharyngeal gut is also differentiating teeth, but the details were difficult to observe.

(4) *Narrow segment with no ciliated band.* Externally, this segment has ventral cilia and a pair of lateral tactile bristles comparable to the two pairs described above. Internally, the differentiating intestine passes through this segment. The lumen of the anterior part of the intestine is relatively large and the coelom is becoming well-defined (shown more anteriorly in Fig. 39). The beating cilia occasionally observed internally in this area originate from the cells lining the gut.

(5) *The anal segment or pygidium.* The pygidium is a relatively long segment, narrowing to the posterior tip of the larva. It bears the telotroch (Figs. 22, 23, and 40), first described above in connection with the trochophore stage. In favorable preparations of living larvae, a small pair of tactile bristles can be observed at the posterior end (posterior bristles are also pictured by Okada, 1930, for several species of *Autolytus*, and by Malaquin, 1893, for *A. edwarsi*). In the midline between these tactile bristles, at the posterior tip of the larva, is a group of short cilia giving the impression of a minute flattened brush. At least

some of these cilia have their origin anterior to the tactile bristles. Internally, the intestine of the anal segment has not yet differentiated a lumen.

Hatched larvae. Larvae which had developed from several different batches of eggs were kept for several days after hatching, and observations were made on living stages. There was no evidence of setae or tentacular buds, as yet, and no other marked changes were observed.

Comparison of syllid larvae. Most of the descriptions of syllid larvae were written in the 1800s and the first three decades of this century. According to some of the workers of this period the descriptions and figures of others were incomplete and inaccurate. This makes a detailed comparison impossible. However, a few larval characteristics may be compared in a general way. In comparison with other species of the Genus *Autolytus*, *A. fasciatus* is further developed at the time of hatching. According to Agassiz (1863, Plate X, Fig. 2), *A. cornutus* is relatively undifferentiated both externally and internally at hatching, although Malaquin (1893) points out that the absence of cilia in Agassiz's description of this species is probably an error. According to Malaquin, *A. edwarsi* has two lateral pairs of ciliated tufts at hatching but does not develop the equivalent of a prototroch or form other ciliated bands until sometime after hatching, and he makes no mention of cilia covering the ventral surface. In *A. fasciatus* described here, three prominent ciliated areas, as well as cilia covering the ventral surface, are present for some time before hatching. The well developed cilia in this species enable the larva to swim actively and suggest that the larvae are pelagic, for a time at least, after hatching. It has been pointed out that members of other subfamilies of syllids may have a short pelagic existence (Thorson, 1946, *et al.*). Larvae of *A. edwarsi*, in which the formation of cilia is delayed, as compared with the relatively precocious development of cilia in *A. fasciatus*, appear to take up a creeping existence on bryozoans or hydroids almost as soon as they are hatched (Malaquin, 1893). Development of functional mucous cells in *A. fasciatus* suggests that the pelagic life of these larvae may be replaced shortly by a creeping existence with the ability to form slime tubes. The Exogoninae, on the other hand, appear to have no larval cilia and no pelagic existence (Viguier, 1884; Saint-Joseph, 1887; Malaquin, 1893; Pierantoni, 1903; Thorson, 1946).

Anterior tactile bristles similar to those described above for the larvae of *A. fasciatus* are shown in the drawings of several other species of *Autolytus* (Greeff, 1879; Okada, 1930; Dales, 1951) and can also be observed in certain members of the Subfamilies Syllinae and Eusyllinae (Herpin, 1926). They may appear relatively late, however (as in *Eusyllis*), which is also the case for external cilia (Malaquin, 1893). Three pairs of lateral tactile bristles have been described above for larvae of *A. fasciatus.* Okada (1930) showed paired lateral tactile bristles in several species of *Autolytus*, although Agassiz (1863) and Dales (1951) do not show them for *A. cornutus* (described as *A. prolifer* by Dales—see Pettibone, 1954). Paired lateral tactile bristles have also been demonstrated in certain Syllinae and Eusyllinae but not in others (Malaquin, 1893; Herpin, 1926). Apparently larvae thus far examined in the Subfamily Exogoninae lack these tactile bristles, as well as any marked external ciliation. Although tactile bristles are small and delicate so that their presence may have been missed by some investigators, they may prove to be a useful characteristic in the identification of syllid larvae.

As described above, the pharynx of hatching larvae of *A. fasciatus* has two anterior teeth and an anterior and a posterior circle of teeth. Investigations thus far indicate that larvae of the Subfamily Exogoninae lack pharyngeal teeth except for a single median anterior tooth (this is consistent with the structure of the adult pharynx—see Fauvel, 1923). Herpin (1926) described and pictured a circle of differentiated teeth (composed of dorsal and ventral halves) in the larval pharynx of *Odontosyllis ctenostoma* (Subfamily Eusyllinae). These may be comparable to the anterior circle of teeth described above for *A. fasciatus*. Malaquin (1893) does not mention a second circle of pharyngeal teeth in the larvae of *A. edwarsi;* however, a second circle of teeth is indicated in several of his drawings (see Plate XIV, Figs. 17a, 17b especially, and 18). Thus, the posterior circle of pharyngeal teeth may be a specific characteristic of certain members of the genus *Autolytus*. These posterior teeth may have been missed by several investigators, for Dales (1951) points out that larval pharyngeal teeth may be difficult to see. Should their specificity prove true, pharyngeal teeth may serve as a useful criterion for distinguishing between species of syllid larvae as they have between certain other polychaete larvae (Allen, 1959, *et al.*).

The larval stages of *A. fasciatus* which were studied had not yet developed setae and were still in the monopharyngeal stage. It will be necessary to study later stages before setal types and larval segmentation can be compared with other syllids.

The writer wishes to acknowledge the assistance of Mrs. Darthea B. Keith in making the histological preparations of serial sections.

SUMMARY

1. A series of developmental stages, from undivided eggs through swimming larvae, were isolated from the brood pouches of the polychaetous worm, *Autolytus fasciatus* (the red-banded *Autolytus*), collected in Puerto Rican waters. Observations on both living stages and on fixed and stained preparations have been described and photographed.

2. The diploid chromosome number for this species was determined as 12. Fertilization occurs at metaphase I. The cleavage pattern is spiral and involves polar lobe formation. Gastrulation is by epiboly. The trochophore and later larval stages show a precocious development of active cilia as compared with other syllid larvae. Characteristic differentiations of larvae at hatching are anterior and lateral tactile bristles, anterior and ventral mucus-secreting cells, and an anterior pair and two circles of pharyngeal teeth. Larvae were compared with other syllid larvae as regards ciliation, tactile bristles, and pharyngeal teeth.

3. Insofar as the writer knows, the present study is the only one recorded in the literature on the early developmental stages of *Autolytus fasciatus,* and the only one on the Subfamily Autolytinae which includes a series of developmental stages from uncleaved eggs through various larval stages. Much more embryological work needs to be done on the Family Syllidae.

LITERATURE CITED

[illegible] A., 1863. On alternate generation of Annelids and the embryology of *Autolytus cornutus*. *J. Boston Soc. Nat. Hist.*, **7**: 384–409.

Allen, M. J., 1957a. The breeding of polychaetous annelids near Parguera, Puerto Rico. *Biol. Bull.*, **113**: 49–57.

Allen, M. J., 1957b. Histochemical studies on developmental stages of polychaetous annelids. *Anat. Rec.*, **128**: 515–516.

Allen, M. J., 1959. Embryological development of the polychaetous annelid. *Diopatra cuprea* (Bosc). *Biol. Bull.*, **116**: 339–361.

Allen, M. J., 1961. Histochemical observations on the early developmental stages of the polychaete, *Autolytus ornatus*. *Amer. Zool.*, **1**: Abstract 194.

Barrois, J., 1877. Sur quelques points de l'embryologie des Annélides. *C. R. Acad. Sci. Paris,* **85**: 297–299.

Dales, R. P., 1951. Observations on the structure and life history of *Autolytus prolifer* (O. F. Müller). *J. Mar. Biol. Assoc.*, **30**: 119-128.

Fauvel, P., 1923. Polychètes errantes. *Faune de France, Paris*, **5**: 1 488.

Galloway, T. W., and P. S. Welch, 1911. Studies on a phosphorescent Bermudan Annelid, *Odontosyllis enopla* Verrill. *Trans. Amer. Micr. Soc.*, **30**: 13 39.

Gidholm, L., 1964. *In:* Zool. Bidr. Uppsala, in press.

Gravier, C., 1923. La ponte et l'incubation chez les Annélides polychètes. *Ann. Sci. nat. Zool. Paris, ser. 10*, **6**: 153–247.

Greeff, R., 1879. Ueber pelagische Anneliden von der Küste der canarischen Inseln. *Zeitschr. wiss. Zool.*, **32**: 237–283.

Hartman, O., 1945. The marine annelids of North Carolina. Duke Univ. Marine Stat., Bull. no. 2: 1–54.

Herpin, R., 1923. Un essaimage en plein jour d'une Annélide polychète: *Pionosyllis lamelligera*. *C. R. Acad. Sci. Paris,* **177**: 355–357.

Herpin, R., 1924a. Essaimage et développement d'un Eunicien et d'un Syllidien. *C. R. Acad. Sci. Paris*, **179**: 1431–1433.

Herpin, R., 1924b. La ponte et le développement chez quelques Néréidiens et Syllidiens. C. R. Ass. Franc. Sci. Paris, Congrès de Bordeaux, 47th Session: 558–562.

Herpin, R., 1926. Recherches biologiques sur la reproduction et le développement de quelques Annélides polychètes. *Bull. Soc. Sci. Nat. l'ouest France, ser. 4*, **5**: 1–250.

Malaquin, A., 1893. Recherches sur les Syllidiens. Morphologie, Anatomie, Reproduction, Développement. Mém. Soc. Sci. Arts, Lille: 1–477.

Mead, A. D., 1897. The early development of marine annelids. *J. Morph.*, **13**: 227–326.

Müller, M., 1855. Ueber *Sacconereis Helgolandica*. Arch. Anat. Physiol. Wiss. Medicin, Taf. II und III: 13–22.

Oersted, A. S., 1845. Ueber die Entwicklung der Jungen bei einer Annelide, und über äusseren Unterschiede zwischen beiden Geschlechtern. *Arch. Naturg.*, **11.1**: 20–23.

Okada, Yô K., 1930. A remark on the constitution of larval syllids. *J. Mar. Biol. Assoc.*, **16**: 479–487.

Pagenstecher, H. A., 1863. Untersuchungen über einige niedere Seethiere aus Cette. *Exogone gemmifera* und einige verwandte Syllidien. *Zeitschr. wiss. Zool.*, **12**: 265–311.

Pettibone, M. H., 1954. Marine polychaete worms from Point Barrow, Alaska, with additional records from the North Atlantic and North Pacific. *Proc. U. S. Nat. Mus.*, **103**: 203–356.

Pettibone, M. H., 1963. Marine polychaete worms of the New England region. 1. Families Aphroditidae through Trochochaetidae. U. S. Nat. Mus., Bull. 227, Part 1: 1–356.

Pierantoni, U., 1903. La gestazione esterna. Contributo alla biologia ed alla embriologia dei sillidi. *Arch. zool.*, **1**: 231-252.

Potts, F. A., 1911. Methods of reproduction in the Syllids. *Ergeb. u. Fortschr. Zool.,* **3**, Heft 1: 1–72.

Raven, Chr. P., 1958. Morphogenesis: The Analysis of Molluscan Development. Pergamon Press, New York.

SAINT-JOSEPH, BARON A. DE, 1887. Les Annélides polychètes des côtes de Dinard. *Ann. Sci. Nat., part 1, ser. 7,* **1** : 127–270.

SCHNEIDER, J., 1914. Zur Entwicklung der *Pionosyllis pulligera* Langerhans. *Zool. Anz.,* **44** : 621–627.

SNOW, R., 1963. Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. *Stain Tech.,* **38** : 9–13.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the Sound (Ørésund). *Medd. Komm. Danmarks Fisk.-Havundersøgelser, Ser.: Plankton,* **4**, no. 1 : 1–523.

VIGUIER, C., 1884. Études sur les animaux inférieurs de la baie d'Alger. Sur l'*Exogone gemmifera* (Pagenstecher) et quelques autres Syllidiens à gestation. *Arch. Zool. Exp. Gén. Paris, ser. 2,* **2** : 69–110.

WILSON, E. B., 1904. Experimental studies on germinal localization. I. The germ-regions in the egg of *Dentalium*. *J. Exp. Zool.,* **1** : 1–72.

A COMPASS DIRECTIONAL PHENOMENON IN MUD-SNAILS AND ITS RELATION TO MAGNETISM[1]

FRANK A. BROWN, JR., H. MARGUERITE WEBB
AND FRANKLIN H. BARNWELL[2]

Marine Biological Laboratory, Woods Hole, Mass., and the Departments of Biology, Northwestern University, Evanston, Ill., and Goucher College, Towson 4, Md.

The tendency of the mud-snail, *Nassarius,* to veer away from an initial southward path, in the earth's magnetic field, displays both a solar-daily and a lunar-daily rhythmic component. It was demonstrated (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960) that an increase in intensity of the horizontal component of magnetic field from the earth's natural one, 0.17 gauss, to 1.5 gauss increased the veering tendency. The increased turning in response to experimentally augmented field also showed, parallelly, both solar and lunar daily variations and their derivative by periodic interference, the synodic month. The south-directed snails were able, furthermore, to distinguish between 1.5-gauss horizontal fields which were parallel to their body axis and to the earth's field and those which were at right angles to them both (Brown, Bennett and Webb, 1960; Brown, 1960).

In some preliminary experiments it was found also that *Nassarius,* while being held in a uniformly illuminated field, appeared to distinguish among the four compass orientations, north, east, south and west, and to exhibit a mean path characteristic for each of these directions (Brown and Webb, 1960). This compass-directional phenomenon seemed to possess a monthly modulation. The pattern of differences of turning for the four compass directions during the fortnight centered on full moon, in each of two consecutive months, was of large amplitude and unimodal with maximum right turning when north-directed and minimum when south-directed. For the alternate fortnights, those centered on new moon, the pattern was bimodal and of approximately half the amplitude, with maxima in right turning when either north- or south-directed and minima when east- or west-directed.

Experiments with the planarian worm, *Dugesia* (Brown, 1962a), have revealed a comparable compass-directional phenomenon with right-turning when the worms are directed either northward or southward and left-turning when directed either eastward or westward. The horizontal vector of magnetism was shown clearly to be involved in this response by experiments in which a horizontal bar magnet, producing a 5-gauss field at the level of the worms, was rotated beneath the orienting worms. The worms behaved as would have been expected

---

[1] This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University (1228-03), and by grants from the National Science Foundation (G-15008) and the National Institutes of Health (RG-7405).

[2] The authors wish to acknowledge their indebtedness to Catherine Jenner, Rinda Metz, Shayna Johnson, Nell Lillie, and Joan Nutt for helping to obtain the data in this report.

had the apparatus itself simply been rotated in the earth's field. However, to a rotated 10-gauss horizontal field the resulting pattern was essentially the inversion, or mirror-image, of that observed for the 5-gauss and for the earth's natural one.

The following experiments were designed to determine more decisively whether mud-snails actually do exhibit such a compass-directional phenomenon in the earth's field, and if so, to learn more concerning its characteristics, including its degree of reproducibility or variability and whether it is, as proven for the worms, dependent at least in part upon response to magnetism.

Methods and Materials

Mud-snails, *Nassarius obsoletus,* were collected every few days from Chapoquoit beach on Buzzards Bay near Woods Hole, Massachusetts. The apparatus was essentially the same as that employed in the earlier published experiments (Brown, Brett, Bennett and Barnwell, 1960). There were, however, two differences. First, the visual field into which the snails emerged from their corral was, among the experiments, in some cases symmetrical and in others asymmetrical with a black background to the left and a white one to the right. Second, the sectors of the grid for recording the clockwise or counterclockwise deviations of individual snail paths at the end of three centimeters of free movement were 11.25° ones for the experiments conducted in 1961 and 1962 instead of the 22.5° ones employed in 1959 and 1960. However, as in the earlier experiments one sector was centered directly ahead of the corral exit (0°). The experimental observations consisted of ascertaining the amount of turning of snails as either the whole apparatus or, instead, simply a horizontal experimental magnetic field was rotated.

Orientation of apparatus in the earth's field. The data on the relationship between geographic direction of the snails' initial orientation and their subsequent turning tendency were obtained from four experiments. In three of these experiments series were run always in quadruplicate, involving four observers using four identically constructed pieces of apparatus. The fourth experiment was run in duplicate.

Experiment I was done during the summer of 1960 on 43 different days between June 30 and August 30. In this experiment the snails emerged from the funnel-shaped neck of their corral into a symmetrical, white field. Five snails emerged in each of the four compass directions, north, east, south, and west, and then the series was immediately repeated in the same sequence. The average path of the ten snails for each direction was determined. The observations were made always between 1:00 and 3:30 P.M.

Experiments II and III were both performed during the summer of 1961. The conditions of these two experiments differed from one another and from the conditions of the 1960 experiment. The snails now emerged into an asymmetrical visual field with a black background to left and a white one to right. The mean paths of all snails in both these experiments favored clockwise turning; the snails were, on the average, positive in phototactic response.

Experiment II, totalling 17 quadruplicate series, involved two series obtained each week between June 22 and August 17, inclusive. Between June 22 and July 6 the mean path for each of the four geographic directions was obtained from series in which additional experimental variables were interpolated (for another purpose) between the successive simple directional ones. Each series comprised emergence of snails under each of 13 conditions: N-directed, two experimentally modified magnetic conditions; E-directed, the same two modified magnetic conditions; S-directed, the two modified fields; W-directed, the modified fields; and finally N-directed in a reversed asymmetrical field. For each daily series, conducted always between 1:00 and 3:30 PM, five snails were permitted to emerge from the corral under each of the 13 conditions, and then, immediately, five snails emerged under each of the 13 conditions in the reversed order of the series. From these series the values obtained for the N-, E-, S- and W-directed samples were isolated and used in the analysis presented in this report. From July 11 through August 17, the series was simplified to the four compass directions alone, with five snails emerging in each of the four directions in a clockwise order (N, E, S and W); then, at once, the series was repeated in the reversed order. As with Experiment I these data were reduced immediately to the mean path for the samples of 10 snails for each direction. The data obtained after July 10 appeared to yield a pattern consistent with those obtained for the period before this date.

Experiment III comprised 25 quadruplicate series obtained during the period of June 21 through August 18, usually on three days of each week. A series consisted of recording five snail-paths under each of a sequence of 14 conditions and, at once, the paths of five snails under the same conditions but in reversed order. Again, the experiment was conducted always between 1:00 and 3:30 PM. The conditions in this group and their serial order were: N, NE, E, SE, S, SW, W, NW and five magnetically modified fields followed by a N-directed, reversed asymmetrical visual field. The last six conditions were included for another purpose. From this series the results obtained for the N-, E-, S- and W-oriented samples were isolated and treated separately for comparison with the data from Experiments I and II. However, the data of all eight compass directions were also analyzed as a unit, and the results were compared with those of a later experiment employing the same eight directions.

Experiment IV comprised directional observations made in 1962 in association with a study of response to alterations in electrostatic fields. Series were run on 27 afternoons between June 20 and August 17, inclusive. Two observers each recorded the paths of five snails for each direction as the apparatus was rotated from N to NE to E to SE. For each direction the snails were subjected first to equipotential plates to right and left followed by a 2-volt/cm. gradient in the air with the positive plate first to right and then to left. The order of the last two conditions was alternated from one series to the next. Immediately upon completion of the series, the order was traversed in the reverse sequence, again with five snails for each condition. Meanwhile two other observers were carrying out observations with fully comparable series but for the directions S to SW to W to NW, and the reverse. From these series the mean paths for each of the four directions, N, E, W and S, with the equipotential fields were isolated and compared with the data for the preceding two summers. The data for the entire eight directions were also considered in comparison with those eight obtained in 1961.

Rotation of an experimental horizontal magnetic field. In a second kind of experiment, V, snails were observed as they emerged from the corral in apparatus oriented so that the organisms always moved initially north but with different orientations of an experimental, horizontal magnetic field. This experiment was always performed by four observers working concurrently with four sets of apparatus. A total of 26 quadruplicate series were recorded between June 21 and August 18, 1961. The series were always obtained in the morning between 8:30 and 11:00, three days a week. Each experimental series comprised recording the path of snails in a series of conditions as follows: (1) controls in the earth's natural field, (2) horizontal experimental 5-gauss fields with S-pole of the bar magnet directed in each of eight compass directions. The sequence in the series was on each occasion as follows: (1) control; (2) 0° (N); (3) 45° (NE); (4) control; (5) 90° (E); (6) 135° (SE); (7) control; (8) 180° (S); (9) 225° (SW); (10) control; (11) 270° (W); (12) 315° (NW); and (13) control. The paths of five snails were determined for each condition from 1 through 13, and then, after assaying the paths of 10 snails in a reversed asymmetrical field, passing back through the series from 13 to 1. By this procedure, controls in the two-way series both immediately preceded and followed each experimental magnetic orientation. The mean path of the 10 snails for each experimental condition was then determined and expressed as the difference from the mean of all control paths.

A double experiment: Rotation of apparatus and rotation of 5-gauss field. Additional experiments were done during mornings of 1962 from June 21 through August 18. One of these, Experiment VI, resembled Experiment IV except that it was conducted between the hours of 8:30 and 11:00 AM instead of 1:00 and 3:30 PM. Twenty-six duplicate series of 8 compass directions were thus obtained by four observers working concurrently and cooperatively. On alternate mornings, similarly usually three days a week, 26 quadruplicate series (Experiment VII), were obtained as follows. The snails were always directed magnetic north but were subjected, *in shuffled order,* to 16 conditions. Twelve of these involved horizontal fields of 5 gauss with the south pole of the bar magnet oriented in 12 compass directions; for the remaining four conditions the experimental field was removed completely so as to provide controls in the earth's field. The twelve directions of the 5-gauss field were 0°, 22.5°, 45°, 67.5°, 90°, 112.5°, 135°, 157.5°, 180°, 225°, 270° and 315°. As in other series, the 16 conditions were assayed by five snail paths first in one order and then in reversed order. The mean path for each of the compass orientations was then expressed as the difference from the average path for all controls in the same series.

RESULTS

I. *Apparatus rotation in the earth's field*

a. *Four compass directions.* The relationship between turning tendency of snails during the first three centimeters of free movement after emergence from a corral exit and the compass direction in which the exit was directed was determined in the following manner from the data of the afternoon experiments I for 1960, II and III for 1961, and IV for 1962 and the morning group VI for 1962. The mean path of all snails for the two or four replicate series for the particular afternoon or morning for all four or eight directions was first determined. Then the mean path for each of the four directions, N, E, S and W, was expressed as the difference from the mean path for all the directions. The average differences for all the series in each experiment are plotted separately in Figure 1.

For experiments I, II and III a general similarity is evident both in form

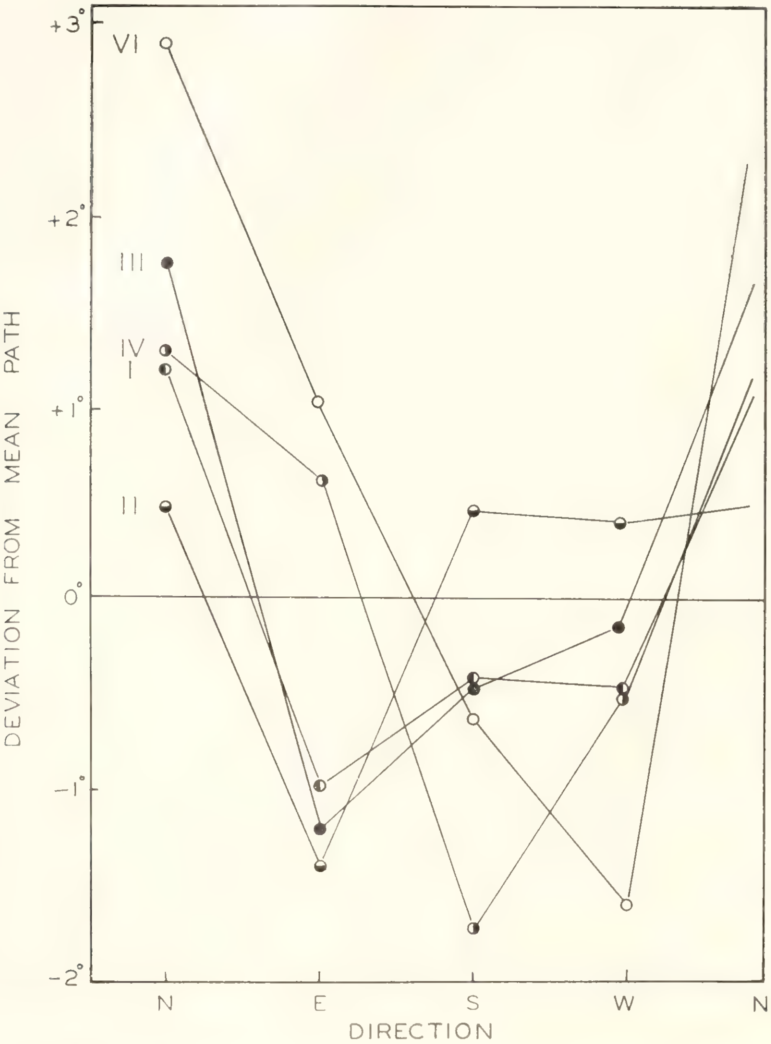


Figure 1. The variation in mean path of snails, in a uniform pattern of illumination, with the compass direction of the initial path. Roman numerals refer to the experiments described in the text.

and amplitude in the variation in mean path with compass direction. This is true whether the mean path of all the snails averaged close to 0°, as in the symmetrical fields employed in 1960, or averaged about +16°, as in the asymmetrical fields employed in 1961. However, the form of the compass relationships for the 1962 snails of Experiment IV, obtained at the same time of day as the earlier group but in the equipotential fields in the morning, differed somewhat from those seen for Experiments I, II and III.

When the differences between paths for the E-directed and N-directed snails were examined, a non-parametric comparison of the 43 samples of Experiment I revealed 28 negative, 14 positive and 1 zero ($P < 0.05$). A similar comparison for Experiment II yielded 12 negative and 5 positive ($P < 0.1$), and for Experiment III 18 negative, 4 positive and 3 zero ($P < 0.005$). A non-parametric consideration of the combined experiments (58 negative, 23 positive, 5 zero) yielded $X = 15.1$; $P < 0.001$. For the N to S difference for Experiment IV there were found 28 negative, 22 positive and 2 zero. For experiment VI the N to W difference showed 34 positive, 17 negative and 1 zero ($P < 0.02$).

b. *Monthly rhythms in north-to-east path difference.* Despite substantial variability within each of the four afternoon groups there appeared to be a reasonably reproducible, significant mean compass-directional response for the snails in the earth's natural field. In view of the preliminary suggestion that this directional phenomenon displayed a monthly variation (Brown and Webb, 1960), the data were examined for the existence of such a periodism. The differences between the paths of E- and N-directed snails for Experiments I, II and III are plotted against calendar date in Figure 2A. The calendar dates for 1960 have been displaced along the abscissa, relative to those for 1961, in order that the three groups be phase-synchronized with respect to elongation of the moon. The days of new moon are indicated.

It is evident from inspection of Figure 2A that a large measure of the observed variability in the relationship between compass direction and snail path is attributable to a synodic monthly variation. The large N to E difference illustrated in Figure 1 for 1960 and 1961 is most striking for the periods over full moon and least so for the periods over new moon. At this latter time there is an evident tendency for this directional relationship even to reverse sign. Eighteen of the 23 cases of the reversal indicated earlier are seen to be accounted for during 15-day periods centered on new moon.

It is also noteworthy that in passing from late June and early July to late August there is for each of these two years a gradual trend toward reduction in amplitude of the directional phenomenon. The data also suggest that when the effect is strongest, as in early summer, there is a tendency for (1) assumption of a semi-monthly variation, and (2) occasional single-day inversions, *e.g.*, July 14 and 19, 1960. Such abrupt inversions appear conspicuously to be followed the succeeding day by unusually strong effects in the more typical direction for the period.

An examination of the corresponding parameter for the data of Experiment IV, obtained at the same time of day, but in 1962, revealed that there was not a monthly but rather a semi-monthly variation in the north to east difference. This variation is illustrated in Figure 2B where the path of the east-directed, relative to the north-directed snails, is plotted in relation to the days of both new and full moons for the two-month period. The data illustrated in Figure 2B include all 27 days investigated in 1962. The data were rather uniformly distributed over four consecutive semi-months of the summer. For the first semi-month there were 7 days of data, ranging from 11 days before new moon to three days afterward. For the second semi-month there were also 7 days, ranging from 11 days before full moon to three days afterward. The third semi-month included 7 days, ranging from 8 days before new moon to three days afterward, and the fourth semi-month contained 6 days, ranging from 9 days before full moon to two days afterward.

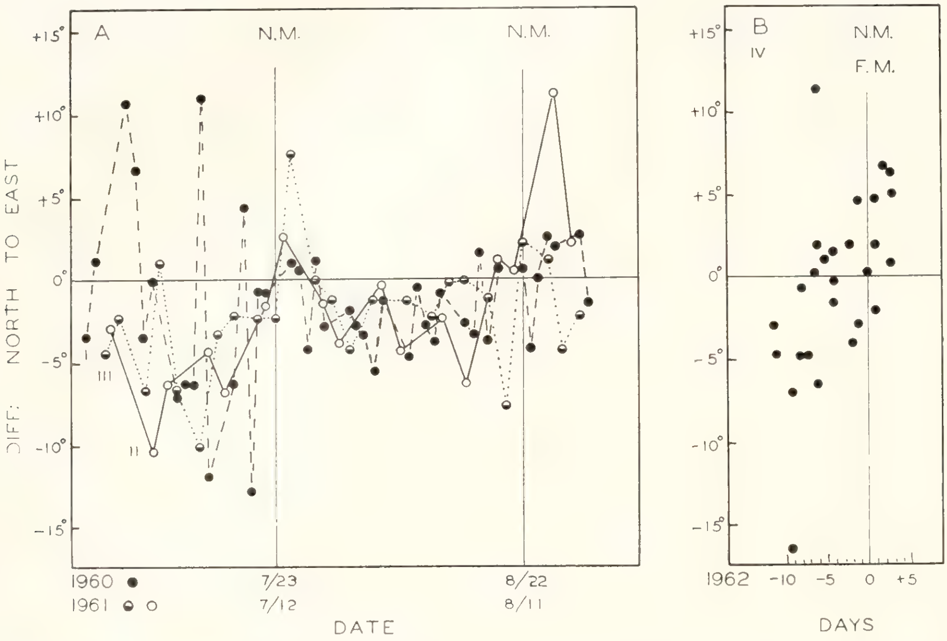


FIGURE 2. A. The variation in difference between the mean paths of E-directed snails and N-directed ones with calendar date for one experiment, I, in 1960 and two independent experiments, II and III, in 1961. B. The comparable differences for the two-month series of experiment IV conducted in the summer of 1962, with the values now plotted with reference to days of new and full moon.

The relationship changed sign over new moon in 1962 just as it did for the preceding two summers, but now, in addition, there was sign reversal over the time of full moon. The two highly anomalous values in Figure 2B were obtained on June 22 and 25, 6 and 9 days before the early-summer new moon, a period when comparable highly atypical values were obtained during summer of 1960.

c. *Eight compass directions.* It will be recalled that the series of Experiment III, involving rotation of snails in the earth's field alone, included compass directions not only parallel (N and S) and at right angles (E and W) to the horizontal vector of the geomagnetic field, but also directions 45° from these (NE, SE, SW, NW). It is seen in Figure 3A, in which the mean path is plotted against compass direction, that the paths of snails for which magnetic direction deviates by 45° from a parallel or right-angle relationship are in every instance observed to be further to the left than for the adjacent parallel or right-angle fields. It will be noted that the mean paths for all the directions involved right-turning because of the asymmetrical field in 1961.

A quantitative analysis involving all 100 values disclosed the mean path for the directions, NE, SE, SW and NW, to average 1.103° to the left of the mean

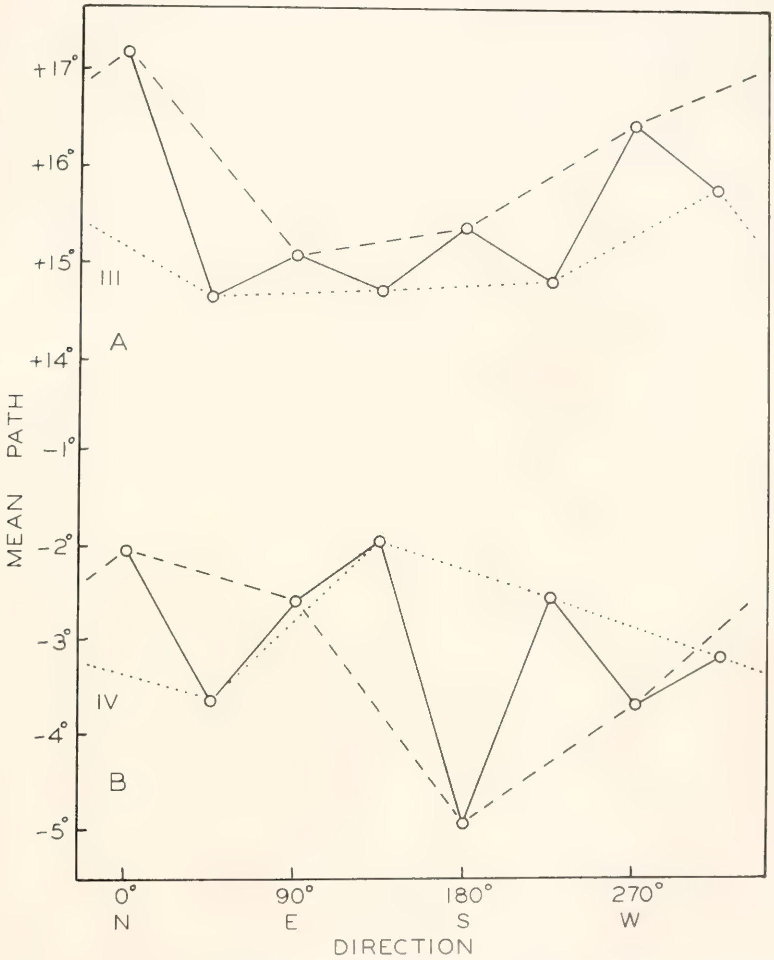


FIGURE 3. A. The relationship between mean path (ordinate) and initial compass direction (abscissa). Snails moved in an asymmetrical light field with a black background to their left. Ordinate values are expressed as deviations from zero which, in turn, represents the direction of initial path. Dashed lines connect the set of points at N, E, S and W; dotted lines connect the set of points for NE, SE, SW and NW. The paths taken for the latter set of directions lie consistently to the left of those taken for the former. B. The comparable relationships in a symmetrical light field and with an artificially produced right-left symmetry of electrostatic field. Note the inversion of relationship between sets of points as initial direction changes from N to SE.

path obtaining for the adjacent directions N, E, S and W. The standard error of the difference was not exactly the same when one computed the differences in paths from those in the adjacent parallel or right angle fields in a clockwise direction on the compass dial as when one computed them from the adjacent ones in a counterclockwise direction. These were ±0.332 and ±0.350, respectively, with $N = 100$. This yielded in either case, however, $P < 0.003$. It seems evident, therefore, that the snails, as indicated by their response, distinguished between parallel or right-angle orientations to the earth's magnetic axis and orientations deviating 45° from these.

When a comparable study was made for all eight directions during two months of the summer of 1962 (Exp. IV) at the same time of day (Fig. 3B) all the comparable relationships appeared to be reversed except for the north to NE and N to NW ones. The mean paths for the SE and SW directions, in consequence, appeared similar to the path for north. The only known and controlled differences between the conditions of the 1961 experimental series shown in Figure 3A and of the 1962 series of Figure 3B were that the field of emergence (1) was asymmetrical in 1961 and symmetrical in 1962, and (2) included the natural ambient electrostatic right-left gradients in 1961 and an experimental equipotential right-left one in 1962. It is interesting that despite the symmetrical field, the mean paths for all directions in 1962 favored slight left turning.

II. *Rotation of a 5-gauss field*

The effect of rotation of a 5-gauss horizontal experimental field (Exp. V, 1961) was determined by computing for each day the mean path of the 40 snails of the quadruplicate sample for each compass direction of orientation of the south pole of the magnet and expressing these as deviations from the mean path for the 200 control snails in the series of the same day. In comparison with the compass directional phenomenon in the earth's natural field, directing the south pole of the bar magnet to the east was the magnetic directional equivalent for the snails of being westbound in the earth's field, and directing the south pole to the west was the magnetic equivalent of a natural eastward path. In other words, clockwise rotation of the experimental magnetic field is the magnetic equivalent for the organism of counterclockwise rotation of the apparatus in the earth's field.

In Figure 4A is plotted the mean path of the snails for each of the magnetically simulated geographic directions, expressed as path difference from all controls in the same series. In the same figure are seen the results for Experiments I and II of rotation of the snails in the earth's field alone. The relationship of response to magnetically simulated direction is seen to approximate closely an inversion of the comparable relationship for the earth's field. Just as for the natural compass-directional phenomenon, the largest difference occurs between N and E, or between the N-directed and W-directed 5-gauss experimental fields. The difference in paths between N and E equivalent magnetic orientations was found to be $1.245 \pm 0.385°$ ($t = 3.23$; $N = 26$, $P < 0.005$).

Experiments VI and VIII were fully independent ones, performed on alternating mornings of the week. Here it is possible to compare for the same time of day for the same two-month period the results of rotating the apparatus in the earth's field with the results of rotating a 5-gauss horizontal experimental magnetic field to simulate the geographical rotation. Both experimental series were similar, too, in being obtained in an equipotential, right-left field across the organism. The two series differed, however, in that the compass directional study was one of an orderly clockwise and then counterclockwise rotation while the experimental magnetic orientations were presented in shuffled order.

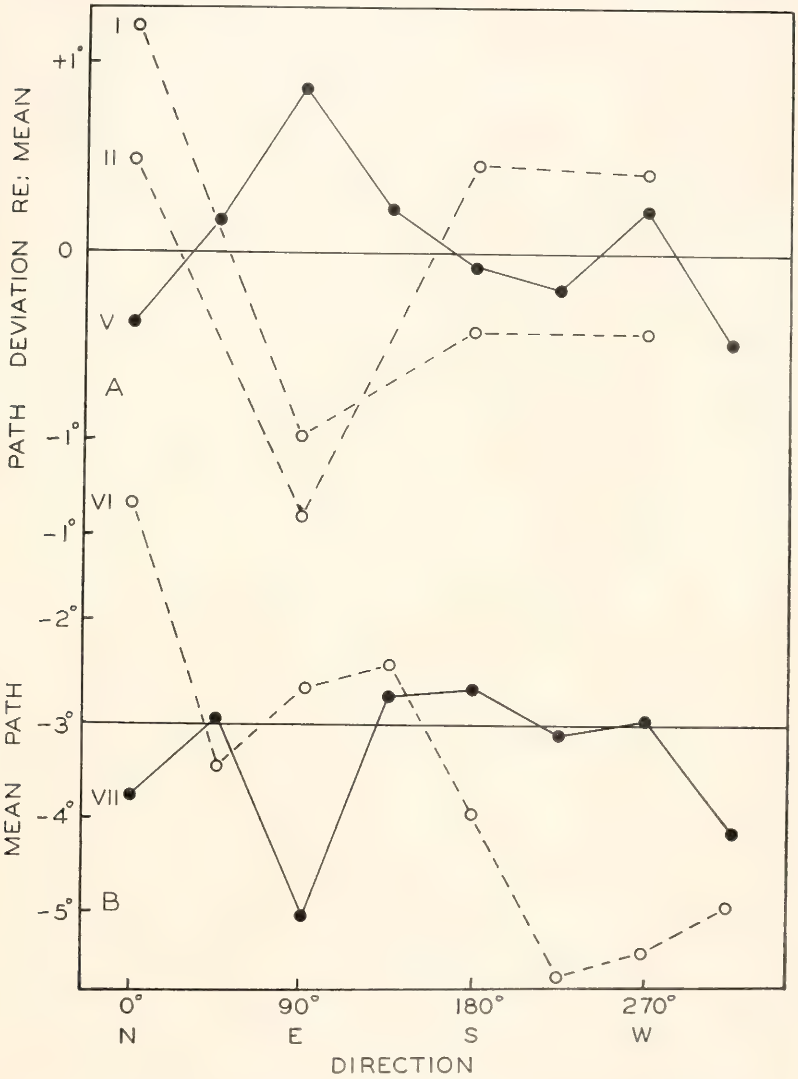


Figure 4. A. Comparison of the pattern of path variation with compass rotation of the initial path in the earth's field, experiments I and II (broken line), and with rotation of a 5-gauss horizontal field, experiment V (solid line), in the opposite direction. B. Same as in A, experiment VI (broken line) and experiment VII (solid line). See text for experimental conditions.

In Figure 4B the mean snail-paths are plotted as a function of both compass direction in the earth's field, and of rotation of a 5-gauss field while the snails were directed steadily northward. The values for the experimental magnetic fields have been plotted as the simulated compass directions as for Figure 4A.

It is evident from Figure 4B that just as had been seen for the experiments in 1961, the snails appeared to be responding to the rotating 5-gauss magnetic field but were exhibiting a response of slightly lower amplitude and in the opposite direction to that observed for the snails during the same period in the earth's 0.17-gauss field. This opposite relationship between response to the natural and the artificial fields obtained for both years, therefore, despite the fact that the two-month periods of 1961 and 1962, with their differing experimental conditions and times, each had its own characteristic associated compass directional pattern.

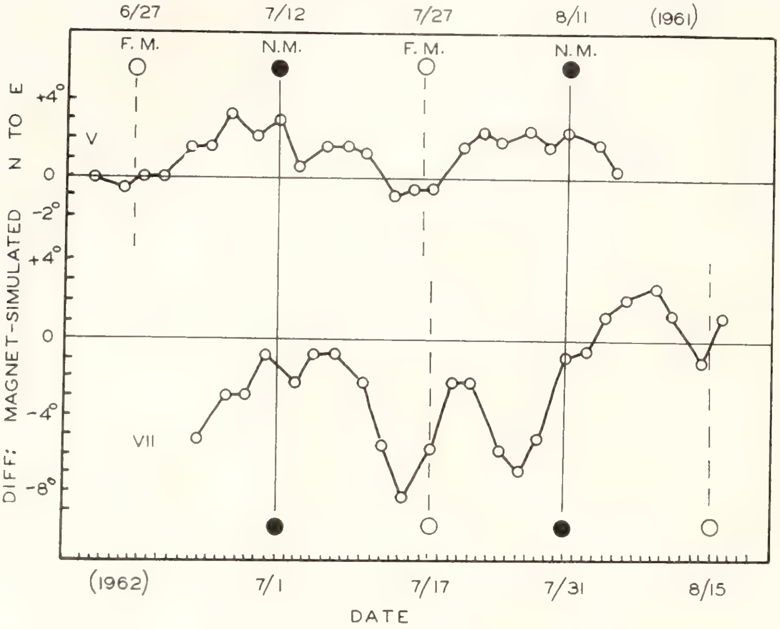


FIGURE 5. Three-day (= weekly) moving means of the difference of the mean path of N-directed snails when in a W-directed 5-gauss field (simulated geographic east) from path with the 5-gauss field N-directed for experiment V in 1961 (upper curve) and for experiment VII in 1962 (lower curve).

As in the case of rotation in the earth's field, substantial variability was encountered in the response to the various compass orientations of the 5-gauss field. It will be recalled that a large portion of the variability in the compass-directional behavior was accountable in terms of monthly or semi-monthly variations. If the snails were using, at least in part, the earth's magnetic field in the directional response, as is strongly suggested by the correlations depicted in Figure 4, it seemed probable that a monthly variation would also be evident for the response to the 5-gauss experimental field. Consequently, the mean path in the east-simulated 5-gauss field was expressed as difference from mean path in the north-directed one.

This gave the 5-gauss counterpart of the N to E difference in the earth's 0.17-gauss field, which was shown in Figure 2 to display a monthly or semi-monthly variation.

In Figure 5 the N to E path differences for the 5-gauss simulated field are plotted together, each expressed as a three-day (= weekly) moving mean to smooth the systematic variations, evident by inspection, in the single-day data. The dates for the two summers have been adjusted to bring into synchrony the days of new moon. The figure reveals a monthly variation for the 1961 experiment of roughly the same general character as that found for the N to E difference in the earth's field alone for the same year (Fig. 2A). A maximum in right-turning tends to center over the time of new moon and minimum over full moon. For 1962 there appears to be a semi-monthly variation as in the compass response in the earth's field for the same year (Fig. 2B). Also quite noteworthy is the relatively small amplitude of the monthly rhythm of 1961 and the much larger amplitude of the semi-monthly one of 1962, holding parallelly for both the orientation in the earth's field and in the 5-gauss simulated fields.

d. *Differences in compass directional pattern with time of day.* Experiments IV and VI, performed in exactly the same manner under the same conditions except for the difference in time of day, permit a comparison of the two to learn whether differences in compass response pattern occur with differing times of day. The morning and afternoon results for the four principal compass directions, plotted together in Figure 1, may be compared. The amplitude and overall gross character of the directional pattern are somewhat similar for the two times of day. However, a difference between them is suggested although the difference is not statistically significant with the available data. Maximum left-turning occurs for the south-directed snails in the morning and for the west-directed ones in the afternoon. That this appears to constitute an alteration in the form of the pattern rather than a phase difference is indicated by the fact that maximum right-turning at both times of day occurs for the north-directed snails.

DISCUSSION

The compass-directional phenomenon clearly indicates a directional heterogeneity in the horizontal plane in which the snails are free to move. It indicates further that the snails must be sensitive to this heterogeneity. The modification of this response by a bar magnet suggests that geomagnetism is involved as one contributing physical factor.

One way to account for the monthly and semi-monthly variations in the directional phenomenon would be to postulate that the two-dimensional pattern of the ambient physical field that the snails perceive is systematically varying in a manner correlated with the rotation of the earth relative to the moon. Alternatively it can be postulated that the lunar rhythms in the directional phenomenon do not depend on monthly variations in particular two-dimensional physical field components by which the organism distinguishes compass directions; instead they may depend on monthly variations in the organism's response to a relatively fixed field pattern. In the latter case the organismic variation could result from a monthly variation in some other physical parameter in the pervasive geophysical environment. The latter factor could even exhibit its variability nondirectionally. Indeed, there could conceivably be an autonomous monthly oscillation within the organism. However. If the last were true the phase angle of the autonomous oscillation must be precisely set by some physical variation correlated with the lunar day, such as illumination or ocean tidal events. This is necessary since for three consecutive summers the monthly orientation rhythm bore fully comparable phase-angle relations to the natural lunar month despite the differing calendar dates for these events.

Regardless of the nature of this cycle timer, the evidence suggests that the factor which determines the phases of the monthly rhythm is related to the 24.8 hour lunar day rather than to the 12.4-hour period of the ocean tides. This seems probable because a tide-correlated factor at Woods Hole, Mass., where the two tides of the lunar day are essentially equal, would be expected to produce semi-monthly rather than monthly cycles. It is difficult to see how a factor cycling every 14.8 days can synchronize a 29.5-day biological cycle with a 29.5-day environmental cycle without 180° phase differences. If, however, tides were actually phasing a monthly cycle, then for 1960 and 1961 the cycles might have been expected to be about 180° out of phase with one another. This is because in 1960 a noon high tide occurred on July 29 six days after a new moon, and in 1961 only three calendar days away on August 1, five days after a full moon. The phase-synchrony of the cycles illustrated in Figure 2A appears therefore to involve some more direct means of lunar synchrony.

In 1962, on August 6, the noon high tide occurred as in 1960, six days after a new moon, and yet the monthly rhythm did not resemble that observed for 1960 despite the repetition of the tidal relationship to elongation of the moon.

It is possible that the monthly phase relations are set solely by monthly variations in nocturnal illumination. More probable, however, would appear to be a response of the snails to some regular variation in the earth's patterns of subtle geophysical forces related to the lunar day. In support of this last hypothesis are the recently demonstrated extraordinary sensitivities of living things to the strengths and vector directions of the earth's magnetic (Brown, 1962a), electric (Brown, 1962b) and radiation (Brown, 1963) fields. Pointing to this, also, are the persistent monthly rhythms, locked in a similar lunar relationship in the flatworm, *Dugesia*, coming from a tideless habitat and held in environments with no access to nocturnal illumination. In the worms, orientational responses appear distinctly phase-synchronized to upper, or both upper and lower, transit of the moon, just as for the snails.

Supporting an hypothesis that different physical parameters are responsible for the two phenomena, (1) distinguishing compass direction and (2) monthly variation in the response to directions, are the observed results of rotating an experimental 5-gauss field, together with the monthly rhythms in response to this stronger field. The phases of the monthly rhythms are essentially synchronized for the two field strengths despite the fact the compass response patterns are the opposite of one another.

It should be emphasized, however, that this particular monthly variation of the snails cannot be attributed exclusively to a completely autonomous monthly periodism of this frequency within the organism. The expression of the rhythm is a function of arbitrarily imposed geographical or experimental magnetic directional changes on the organisms. In other words, the phase angle of one of the two essential components in the rhythm is arbitrarily, and for all practical purposes randomly, presented in time.

The mirror-image relationship that the compass response to the 5-gauss field bears to the comparable response to the earth's field suggests that there is a sign change in the response. Such a sign change has been reported for the planarian, *Dugesia* (Brown, 1962a), but in this latter instance the change occurred between 5 and 10 gauss. For *Nassarius* the sign change must occur at some field strength below 5 gauss.

One additional consideration should be mentioned at this time. This pertains to the relatively large variability in the snail responses. There is reason to believe that this resides chiefly in behavioral variability within and among individuals. This is suggested by the relatively huge variances which are encountered in the sampling for any given experimental condition and time on the one hand, and the relatively orderly and systematic variations in the means about which the individual paths vary. This is the kind of a picture one would expect to encounter if, for example, a rather poor marksman were shooting at a bullseye while the target itself was being gradually and systematically changed in its location. In view of the large variability which characterizes biological phenomena, particularly at the organismic level, it seems most reasonable at present to assume that the physical environmental factors which singly or collectively are involved in these phenomena exhibit less variability than suggested by the responses. If the biological phenomenon in this particular case were adaptive rather than probably non-adaptive, variability would be expected to be vastly reduced.

Irrespective of what factors are responsible for the monthly variation in directional response, this study does provide a fairly strong case for geomagnetism as being the most important single physical force responsible for the two-dimensional heterogeneity used as the basic reference in the compass-directional behavior described in this report. But equally supported is the conclusion that other uncontrolled geophysical variables are capable of influencing greatly the response of the organism to the relatively stable average direction and strength of this magnetic field.

SUMMARY

1. Mud-snails in a uniform field of all ordinarily controlled directional factors distinguish among geographic directions.

2. Geomagnetism is involved in this directional sense. This was shown by rotation of a 5-gauss horizontal magnetic field which produced an orientational behavior correlated with that observed when the snails were rotated in the opposite direction in the earth's field.

3. The response pattern to compass directions of the 5-gauss horizontal field was essentially the mirror-image of that observed for the earth's 0.17-gauss field.

4. The directional response of the snails in the geographic field, and the concurrent correlated response to a rotating experimental magnetic field, vary parallelly with time and with influence of other, still undefined factors.

5. Rotation of a 5-gauss horizontal magnetic field through the series of four compass directions, differing by 45° from the four cardinal directions, may produce

a pattern of compass-directional behavior either paralleling or mirror-imaging that observed concurrently for the four cardinal directions.

6. The compass-directional pattern of response of the snails shows a monthly or a semi-monthly variation related to moon phase. There is reason to believe that this is related to the moon through some mediator other than the ocean tides or nocturnal illumination.

7. The compass-directional response to a rotating 5-gauss horizontal magnetic field, shows a monthly variation or a semi-monthly one. Whether it is monthly or semi-monthly, and its amplitude, appear to vary parallelly with comparable cycles obtained during the same period in response to equivalent changes in orientation in the earth's 0.17-gauss field. This gives additional support to the conclusion that geomagnetism plays an important role in these orientational phenomena of the snails.

LITERATURE CITED

Brown, F. A., Jr., 1960. Response to pervasive geophysical factors and the biological clock problem. *Cold Spring Harbor Symp. Quant. Biol.*, **25**: 57–71.

Brown, F. A., Jr., 1962a. Responses of the planarian, *Dugesia*, and the protozoan, *Paramecium*, to very weak horizontal magnetic fields. *Biol. Bull.*, **123**: 264–281.

Brown, F. A., Jr., 1962b. Responses of the planarian, *Dugesia*, to very weak horizontal electrostatic fields. *Biol. Bull.*, **123**: 282–294.

Brown, F. A., Jr., 1963. An orientational response to weak gamma radiation. *Biol. Bull.*, **125**: 206–225.

Brown, F. A., Jr., and H. M. Webb, 1960. A "compass-direction effect" for snails in constant conditions, and its lunar modulation. *Biol. Bull.*, **119**: 307.

Brown, F. A., Jr., M. F. Bennett and H. M. Webb, 1960. A magnetic compass response of an organism. *Biol. Bull.*, **119**: 65–74.

Brown, F. A., Jr., W. J. Brett, M. F. Bennett and F. H. Barnwell, 1960. Magnetic response of an organism and its solar relationships. *Biol. Bull.*, **118**: 367–381.

Brown, F. A., Jr., H. M. Webb and W. J. Brett, 1960. Magnetic response of an organism and its lunar relationships. *Biol. Bull.*, **118**: 382–392.

ADAPTATION OF THE MAGNETORECEPTIVE MECHANISM OF MUD–SNAILS TO GEOMAGNETIC STRENGTH[1]

FRANK A. BROWN, JR., FRANKLIN H. BARNWELL
AND H. MARGUERITE WEBB[2]

Marine Biological Laboratory, Woods Hole, Mass., and the Departments of Biology, Northwestern University, Evanston, Ill., and Goucher College, Towson 4, Md.

The mud-snail, *Nassarius,* has been proven to be sensitive to a magnetic field as weak as 1.5 gauss and to be able to distinguish between fields parallel and at 90° to its path of locomotion, (Brown, Brett, Bennett and Barnwell, 1960; Brown, Webb and Brett, 1960; Brown, Bennett and Webb, 1960). A similar capacity has been reported for *Volvox* by Palmer (1963), who employed 5-gauss fields. An extensive study by Brown (1962) of the planarian, *Dugesia,* established that this worm, too, displayed extraordinary sensitivity to very weak magnetic fields. It was demonstrated that in an unchanging illumination field, the tendency of the worms to veer from an initial direction of movement varied in a manner characteristic of each compass direction of the initial path. The rotation of a 5-gauss magnetic field had qualitatively the same effect on the path taken as did equivalent reorientation of the worms themselves in the earth's field. Qualitatively the same type of response was also evident for the weaker field strengths, 2.0 and 0.25 gauss, but the response became progressively weaker as the experimental field strength approached that of the earth's own horizontal field, 0.17 gauss, which was always present. However, when the field strength was raised to 10 gauss, the normal response disappeared and was replaced by an opposite, or mirror-imaged, compass directional pattern. Upon the basis of preliminary experiments it has also been reported that changing the orientation of a 5-gauss horizontal field gives a response pattern which is the inversion of that observed upon changing the orientation of snails in the earth's field (Barnwell and Brown, 1964).

While the foregoing experiments strongly suggested that the receptor system for organismic response to the horizontal vector of magnetism was one specialized for such weak fields as the earth's, the method of study would not permit resolution of the particular field strength for optimal response. In consequence, the experiments to be reported here were designed and conducted. The results point strongly to the conclusion that the optimal resolving capacity of a living system for the direction of the horizontal component of magnetism is exquisitely adjusted to the strength of geomagnetism.

---

[1] This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University (1228-03), and by grants from the National Science Foundation (G-15008) and the National Institutes of Health (RG 7405).

[2] The authors wish to acknowledge their indebtedness to Helen Scott, Jean Leiberman, Thomas Schroeder, Kathleen Alexander and Louise Skalko for helping to obtain the data of this report.

METHODS AND MATERIALS

The mud-snails, *Nassarius obsoletus*, which were used throughout this study were collected once or twice a week at Chapoquoit beach on Buzzards Bay, a few miles north of Woods Hole, Massachusetts.

The experiments consisted of assaying the turning tendency of the snails moving initially magnetic south in the earth's field and alternating these assays with measurements of the turning tendency of the same snails subjected to an abruptly reversed experimental field. The reversed field was produced by a cylindrical bar magnet placed horizontally beneath the organisms and oriented to oppose the earth's horizontal vector and override it to produce a series of strengths of the reversed field as follows: 0.04, 0.1, 0.2, 0.4, 0.8, 2.0, 5.0 and 10.0 gauss. The experiments were performed over two-month periods during the summers of 1960, 1961 and 1962, with slight differences in the procedure between one summer and the next. In each year, the experiment was performed in quadruplicate, with four observers working concurrently with four identically constructed pieces of apparatus.

The apparatus has been described in some detail elsewhere (Brown, Brett, Bennett and Barnwell, 1960; Barnwell and Brown, 1964).

Experiment I was performed in 1960 during the two-month period from June 29 to August 26, inclusive. Quadruplicate series were run on 47 mornings, between 9:30 and 12 o'clock, distributed as uniformly as was practical over the whole two-month period. For purposes of analysis, the total period was subdivided into two monthly periods. Month 1 included 22 days of observation from June 29 through July 26, and month 2 comprised 25 days of data obtained between July 28 and August 26, inclusive. The snails emerged from the exit of an aluminum, funnel-shaped corral into a symmetrically illuminated field with white backgrounds to both right and left.

Each experimental series consisted of 16 sets of 5 snail paths; a set of 5 paths obtained in the earth's field regularly alternated with a set of 5 paths obtained in a reversed horizontal magnetic field. Eight different field strengths were tested, always in the same order. The pattern of each experimental series was as follows: C (= control); 0.04 (gauss); C; 0.1; C; 0.2; C; etc. Immediately upon completion of these observations the entire series was repeated but in reversed order. Thus, each of the four observers on each morning assayed a total of 10 snail-paths under each of the 16 conditions.

Experiment II was carried out during the summer of 1961. The experiments were conducted, again in quadruplicate, on 26 mornings between 9:30 and 12 o'clock, usually on three alternate mornings a week, between June 22 and August 19, inclusive. For analysis the data were subdivided into month 1, including 13 days between June 22 and July 20, inclusive, and month 2, consisting of 13 days from July 22 through August 19. In these experiments the snails emerging from their corral entered an asymmetrical field of illumination with black to left and white to right. The snails, in general positively phototactic, tended to veer to the right. Each daily series comprised observing the paths of 5 snails emerging under each of 14 conditions; then 5 additional paths were observed under each of the conditions presented in reversed order. The 14 conditions in their initial order were: C; 0.04; 0.1; C; 0.2; 0.4; C; 0.8; 2.0; C; 5.0; 10.0; C; and C with a reversed asymmetrical field. Hence, each observer obtained the paths of ten snails under each of the 14 conditions.

Experiment III was performed on 18 afternoons between 1 and 3:30 o'clock, usually on Tuesday and Thursday each week over the period, June 21 through August 20, 1962. Month 1 comprised 9 days between June 21 and July 19, inclusive, and month 2 included 9 days from July 24 through August 20. In this experiment the snails emerged into a symmetrical field with large aluminum plates to right and left. The plates were interconnected to assure their equipotential condition but were not grounded. The mean paths for 5-snail samples were determined by each of two observers for each of 13 conditions in the following order and then, at once, for the same series in reversed order: C; 0.04; 0.1; C; 0.2; 0.4; C; 0.8; 2.0; C; 5.0; 10.0; and C. At the same time two other observers were proceeding through comparable series which differed only in that the successive pairs of the magnetic fields were reversed (*e.g.*, C; 0.1; 0.04; C; 0.4; 0.2; C; 2.0; 0.8; . . .).

In experiment I, conducted in 1960, each of the sectors of the polar coordinate grid from which the mean snail paths were determined at 22.5°. For experiments II and III during the succeeding two summers, the sectors were reduced in size to 11.25°.

In view of the fact that the response of snails to weak magnetic fields has been demonstrated to display solar-day (Brown, Brett, Bennett and Barnwell, 1960) and lunar-day (Brown, Webb and Brett, 1960) variations and consequent monthly or semi-monthly ones, the data were reduced at once to monthly values. For each of three years there were available four individually obtained series for each of two months—a total of 24 monthly values representing six different months. The influence on the turning tendency of the snails of each of the eight experimental field strengths was computed separately for each of the 24 series of values. Two methods of computation (Methods A and B below) were carried out for each series.

By method A for the 1960 experiment I, the mean path in each strength of reversed magnetic field was expressed as the difference from the adjacent control sample which preceded it as the series was run in one direction and followed it when the order was reversed. By method B the mean path of the snails for each strength of reversed field was expressed as the difference from the mean path obtained by averaging all controls.

Methods A and B for computing the influence of the experimental fields were applied in a comparable manner to the data of the succeeding two summers. For experiment II, in 1961, method A consisted of expressing the effects in terms of differences from the immediately adjacent controls. As a consequence, the members of the pairs of strengths, 0.1 and 0.2, 0.4 and 0.8, and 2.0 and 5.0 gauss, had common controls. Method B consisted of expressing the influences of the experimental fields in terms of differences in paths from the average path for all controls. For experiment III, in 1962, method A consisted of expressing the mean paths of the snails in each experimental field strength as the difference from the mean path in the two control samples flanking it, both before and after, as the series was run first in one order and then the reversed order. Now, two common controls were shared between 0.04 and 0.1, 0.2 and 0.4, 0.8 and 2.0, and 5.0 and 10.0 gauss, and successive pairs shared one control. Method B in 1962 consisted of expressing the mean path at each strength of reversed field as the difference from the average path for all controls.

The two methods of analyzing the influences of the various strengths of the experimentally reversed fields were employed inasmuch as nothing was initially known concerning whether there were any persistent effects following exposure of the snails to the various experimental fields. It must be noted that as far as magnetism is concerned, two kinds of abrupt reversals were being imposed upon the snails. One of these was the reversal at each of a series of strengths of field as the animals were changed from the earth's 0.17-gauss horizontal field to a lower or higher reversed one, and the other was the return of the snails from the lower or higher reversed experimental field back to the earth's natural one.

It was possible that a persisting influence of abrupt field reversal, or abrupt changes in field strength, or even in some combinations of these changes might occur. If such an influence manifested itself as a modification of the path taken by the snails in subsequent tests, the use of adjacent controls to calculate the effects of experimental conditions might give misleading results. Depending on the direction in which the path was modified the results might indicate effects either larger or smaller than the true ones. In view of this second kind of problem, one additional type of analysis was made. This involved study of the mean paths of the snails in the earth's south-directed 0.17 gauss field, employing the data of the controls in the series of experiments I, II, and III. The mean path for each control sample was considered in relation to the average strength of the experimentally reversed fields which immediately preceded it.

This last consideration obviously required the elimination from this analysis of the first five control paths for all three years, inasmuch as these were preceded by no experimentally reversed field. It also required the elimination, for the same reason, of the first five control paths, as the series was commenced in the reversed order in experiments II and III in 1961 and 1962. The time to obtain a sample of 5 snail paths ranged typically from three to five minutes, and hence in this study the effect of an exposure of this duration to an experimentally reversed field was being assayed over an equal period of time immediately following the removal of the experimental field.

RESULTS

Response to reversed fields expressed as difference from adjacent controls: Method A

In Figure 1A the response is plotted as a function of field strength for each of the three years. Response is calculated as the average deviation from the adjacent controls (Method A). Each point represents the average of all tests in a given year. Inspection of Figure 1A suggests a general over-all trend in the deviations in paths from the controls in passing from the weakest reversed field, 0.04 gauss, to the strongest, 10 gauss. Suggested is an apparent tendency toward right-turning in response to reversed fields weaker than the earth's and left-turning in response to reversed fields stronger than the earth's. This is based, of course, on the assumption that all the controls are fully comparable to one another and that there is no persistent influence of the experimental fields.

Of greater statistical significance, however, are the differences among the variances for the eight field strengths for the three-year study. Variance is maximum for the 0.2-gauss field and minimum for the 0.8-gauss one. Employing Hartley's (1943) method of largest F ratio to determine the probability that there is heterogeneity of variance among the eight samples, *F* max. was found to be 1097 ($P <$ 0.05). In Figure 1B are plotted the variances of these mean responses for the eight strengths of reversed field. Included also in Figure 1B is the distribution of variances computed using all 6 monthly mean values obtained over the three years. In both of these instances the maximum tendency of the snails to deviate from the southward path in the earth's 0.17-gauss field in response to abrupt reversal of the

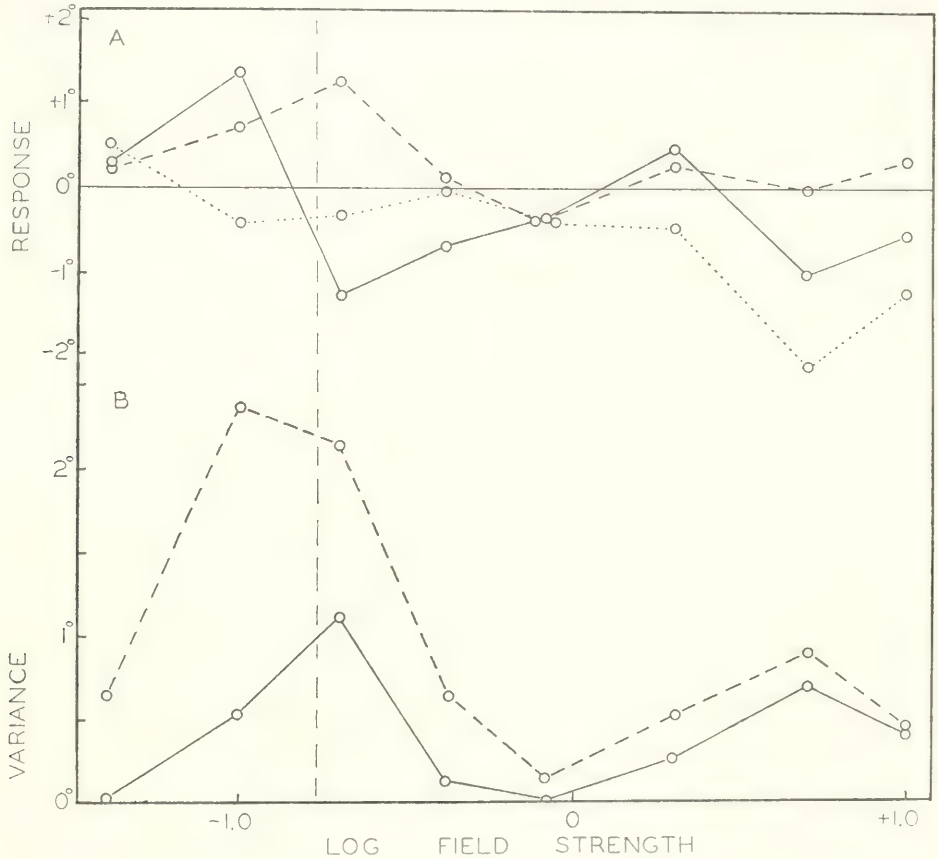


Figure 1. A. The mean deviation in snail path from that of adjacent controls in the experimental series, in response to experimentally reversed magnetic fields at a series of strengths. Solid line, 1960; broken line, 1961; dotted line, 1962. B. The variances obtained for each field strength computed from the three mean annual results (solid line) and from the 6 mean monthly ones (broken line). The vertical dashed line indicates the strength of the earth's horizontal vector.

horizontal vector of magnetism occurs for experimental field strengths closest to that of the natural terrestrial one.

A persisting influence of experimentally reversed fields on succeeding controls

The examination of the controls was next undertaken. In Figure 2 the mean values of controls for each of the three years are plotted in relation to the log of the mean experimental magnetic field strengths that immediately preceded them.

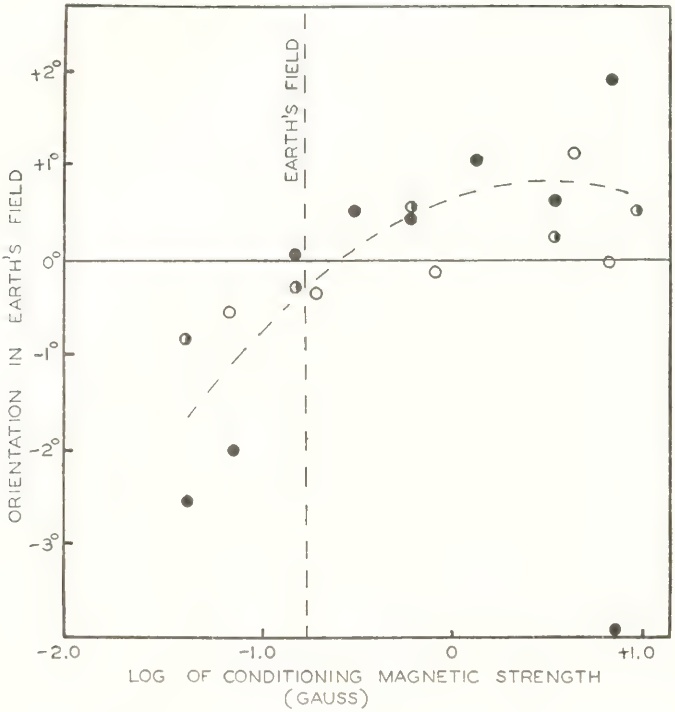


FIGURE 2. The relationship between snail path when southbound in the earth's field and the mean strength of the immediately preceding, experimentally reversed magnetic field. Broken line curve is the computed non-linear relationship. Solid dots, 1960; half-solid dots, 1961; circles, 1962. The values are expressed as differences from the average ones for all strengths for that year.

The controls for each of the three years are expressed as deviations from mean control for that year. It is evident from this figure that the paths of control snails in the earth's field are generally further to the left than the mean following subjection to a reversed field weaker than the earth's and further to right than the mean following subjection to a reversed experimental field stronger than the earth's. Treated as a linear correlation, $r = 0.75$ $N = 18$; $P < 0.001$.

This highly significant correlation indicates that the altered magnetic field exerted an effect that persisted for the three- to five-minute control assay after completion of the test exposure.

When the data depicted in Figure 2 were treated as a non-linear correlation, a better fit was described by the equation

$$Y = 0.631 + 0.695X - 0.696X^2,$$

where X is the log of the field strength expressed in gauss. This relationship is described by the broken line in Figure 2. The non-linear coefficient of correlation was 0.825, indicating that more than 68% of the variation was described by this relationship.

The specific character and magnitude of the magnetic effect persisting in the control snails was more than enough to account for the apparent over-all gradual increase in left-turning in response to increasing strengths of reversed field (Fig. 1A) when these were computed as differences between paths in the reversed fields and paths in adjacent controls.

Response to reversed fields expressed as difference from the mean of all controls: Method B

This method for dealing with the data eliminates any influence of differences among the controls and permits a comparison among the mean paths in the reversed fields of 8 strengths.

In Figure 3A are plotted the relative effects of the eight reversed fields for each of the two months of 1960. The results for the two months display a general similarity to one another. Maximum turning, to the left, for the summer occurs in response to the reversed 0.2-gauss field.

The results obtained for each of the two months of 1961 are illustrated in Figure 3B. Again the results for the two months show a good resemblance to one another ($r = 0.713$); a maximum in turning again occurs at 0.2 gauss, but the direction is opposite that obtained for 1960.

In the experiment performed in 1962, the results obtained for the two months showed a striking difference between them. The results for the two months, for fields weaker than 2 gauss, appeared to mirror-image one another. The results for the first month were strikingly similar to the average of those obtained for the summer of 1960 ($r = 0.805$). The apparent response to the 0.2-gauss field was $-2.25 \pm 0.29°$ ($N = 4$). The results obtained for the second month were more similar to those obtained for 1961.

In Figure 3D are seen the average results for each of the three years, together with selected standard errors. It is evident that the most significant deviation in path from the controls for both 1960 and 1961 occurs at 0.2 gauss with values of $-1.738 \pm 0.526°$ ($N = 8$) and $+0.882 \pm 0.250°$ ($N = 8$), respectively. At this strength of magnetic field were also the most consistent responses, as indicated by the lowest variance in the series of eight field strengths for each of these two years. However, for 1962, as would be expected from inspection of Figure 3C, the response to the 0.2-gauss field exhibits the maximum variance in the series of strengths. The mean path for both months was $-0.750 \pm 0.829°$. The F max. for the 8 individually-obtained series of 1962 was found to be 34.25, indicating a significant heterogeneity of variances among the eight field strengths ($P < 0.01$), with the largest variance at 0.2 gauss and the smallest one at 2 gauss. These results appeared consistent with the results of 1960 and 1961 in showing maximum response to the 0.2-gauss field, but the response, as in these other years together, was either clockwise or counterclockwise turning.

In Figure 4, the relationship between variance of mean paths and strength of reversed field is shown for the 6 months of series of responses for which response

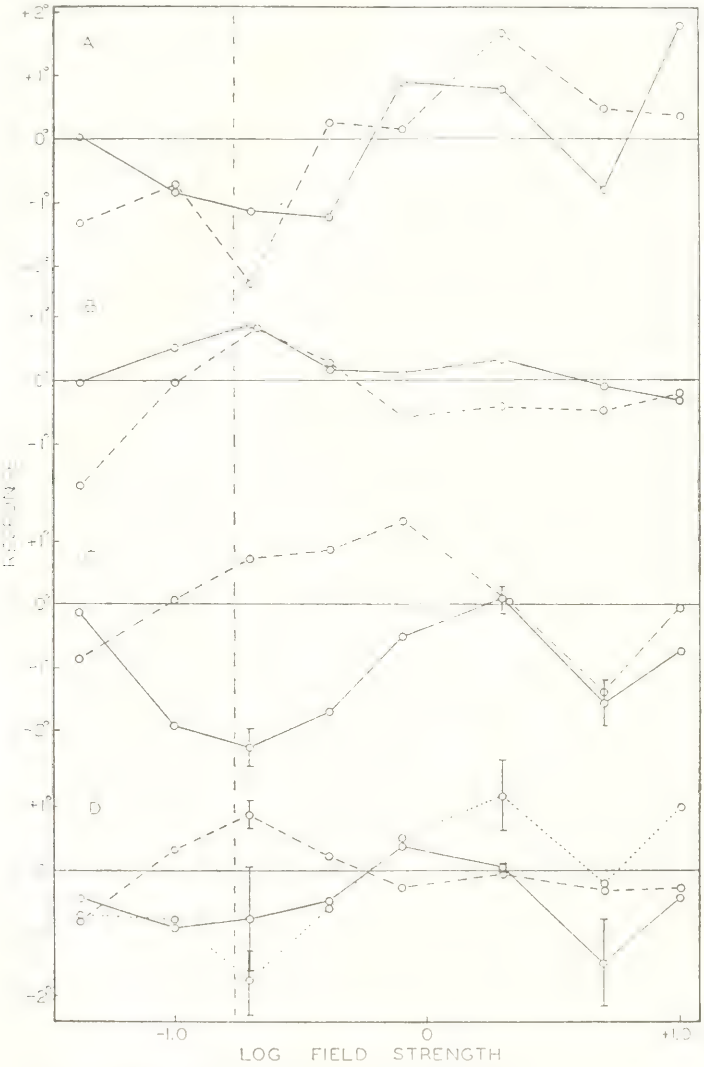


FIGURE 3. The deviation in snail path from the average path for all controls in the series for each of a series of eight strengths of experimentally reversed horizontal vectors of magnetic field. A. Month 1 (solid line) and month 2 (broken line) in 1960. B. Month 1 (solid line) and month 2 (broken line) in 1961. C. Month 1 (solid line) and month 2 (broken line) in 1962. D. The mean annual results for 1960 (dotted line), for 1961 (dashed line), and for 1962 (solid line) expressed as deviations from mean response. Selected standard errors are shown. The vertical dashed line marks the strength of the earth's horizontal component.

was determined as deviation from the average path for all controls in the series (method B). Maximum variance again occurs at 0.2 gauss with a value of 2.014°. Minimum variance, 0.237°, occurs at 0.8 gauss. The ratio of these variances, or F, is 8.5. ($P < 0.01$)

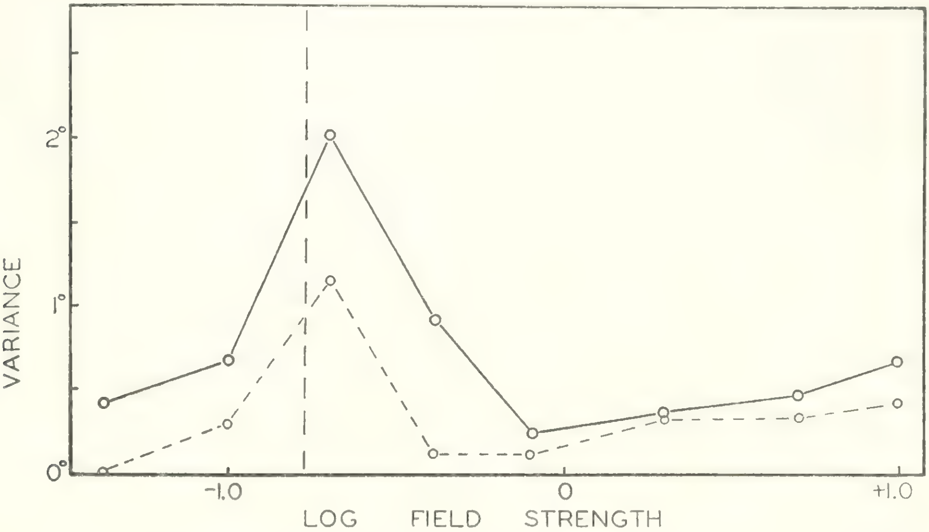


FIGURE 4. The variances obtained for responses to the reversed fields as computed from the data illustrated in Figure 3. The solid curve shows variances computed from 6 mean monthly values, and the broken curve, from three mean annual ones. The vertical dashed line indicates the strength of the earth's horizontal component.

DISCUSSION

Mud-snails initially directed geographic southward display a characteristic mean turning tendency away from this southward path; when directed geographic north, a different mean turning tendency is evident (Brown, Webb and Barnwell, 1964). It was the intent of this investigation to learn at what strength of an abruptly reversed horizontal magnetic vector the snails would be induced to alter to the greatest degree their characteristic "south"-turning tendency.

It is significant of itself that despite the fact that the conditions of the experiment differed over the three summers of study in symmetry of background, in state of the ambient electrostatic field, and in time of day, one relatively uniform result was found. This was that the maximum tendency to alter direction in response to the experimentally reversed horizontal magnetic field appeared to occur when the strength of the reversed field was that one closest in strength to the natural terrestrial one.

On the other hand, it is unfortunate that the variety of conditions used for experiments renders it impossible at this time to reach any similarly well supported conclusion as to why the response should have been for three out of the six months a counterclockwise response, with a clockwise one for the remaining months. That it probably involves the influence of some factor other than any of those involved in the controlled experimental conditions is indicated by the differences observed between the two months of 1962, despite the similarity of all controlled conditions.

The results as described in the relationship between the experimental field strength and the observed variances do, however, indicate a rather sharp adjustment of the receptor mechanism for magnetic compass direction to the strength of the horizontal vector of geomagnetism, *H*, rather than to, for example, the total earth's field strength, *F*, of about 0.5 gauss. It is evident that the responsiveness of the snails has already fallen off greatly even before the horizontal vector strength has risen to 0.4 gauss, and has become minimal at about 0.8 gauss.

It would be difficult to resolve the exact strength for maximum responsiveness to the horizontal vector of magnetism. This difficulty results in part from the variability in strengths and vector directions of these very weak fields that one encounters in and about the ordinary laboratory. Perhaps in far larger measure it results from the discovery, reported here for the first time, of persistent effects of magnetism. These were illustrated in Figure 2. From the figure it is evident that the strength, and possibly even the sign of the apparent response of the snails to terrestrial magnetism, can be altered by a brief conditioning exposure to a slightly stronger or weaker experimentally reversed horizontal field.

Additional and indirect support for the conclusion that maximum capacity to resolve the direction of the horizontal magnetic vector occurs when the simultaneous strength-change has been least comes from Figure 2. The change in sign of the mean response from negative to positive, relative to the mean response for all paths, occurs as the conditioning stimulus strength approaches, and passes through, the strength of the earth's weak horizontal vector.

The results shown in Figure 2 also emphasize the obvious caution needed in any interpretation of studies of magnetic response which may at some future time be carried out in magnetically shielded environments where the ambient field strength is greatly reduced. The response for magnetism appears to resemble other kinds of receptor systems in possessing an ability to adapt or adjust to changing levels. This seems to be true at least within the narrow range of strengths spanned in this investigation.

Such properties as those of the extraordinarily adjusted maximum sensitivity to the level of geomagnetism, even to the specific local horizontal vector strength, and the persistent induced alterations occurring in response to very small field-strength changes lead one to postulate that the mechanism that is concerned in this response is biologically adaptive, rather than a useless, fortuitous property of protoplasmic systems. It appears probable, therefore, that it plays important roles in the lives of the organisms.

SUMMARY

1. The maximum orientational response of the snail, *Nassarius*, to an abrupt experimental reversal of the horizontal vector of geomagnetism occurs when the reversed field deviates least in strength from the earth's horizontal 0.17-gauss one.

2. The responsiveness to field-direction change drops off very substantially even before the field strength has decreased to 0.1 gauss or increased to 0.4 gauss. It is already minimal at 0.8 gauss.

3. The receptor mechanism for compass orientation in response to geographic direction is, therefore, exquisitely adjusted to the strength of the horizontal vector of geomagnetism, even in contrast with the total strength of geomagnetism.

4. There is a persistent effect of experimental magnetic fields deviating in strength from the natural one which remains for at least three to five minutes following removal of the experimental field.

5. Following exposure to reversed horizontal fields stronger than the earth's, southbound snails respond in the earth's field by clockwise turning, and following exposure to reversed fields weaker than the earth's, by counterclockwise turning, relative to their path in the earth's field following exposure to an experimentally reversed horizontal field of the strength of the earth's natural one.

6. These results suggest that the receptor mechanism of the organism for very weak magnetic fields is highly specialized and adaptive, probably playing important roles yet to be disclosed.

LITERATURE CITED

Barnwell, F. H., and F. A. Brown, Jr., 1964. Organismic responses to very weak magnetic fields. Proc. 1st Biomagnetics Symposium. Plenum Press, N. Y. (in press).

Brown, F. A., Jr., 1962. Responses of the planarian, *Dugesia,* and the protozoan, *Paramecium,* to very weak horizontal magnetic fields. *Biol. Bull.,* **123** : 264–281.

Brown, F. A., Jr., M. F. Bennett and H. M. Webb, 1960. A magnetic compass response of an organism. *Biol. Bull.,* **119** : 65–74.

Brown, F. A., Jr., W. J. Brett, M. F. Bennett and F. H. Barnwell, 1960. Magnetic response of an organism and its solar relationships. *Biol. Bull.,* **118** : 367–381.

Brown, F. A., Jr., H. M. Webb and F. H. Barnwell, 1964. A compass directional phenomenon in mud-snails and its relation to magnetism. *Biol. Bull.,* **127** : 000–000.

Brown, F. A., Jr., H. M. Webb and W. J. Brett, 1960. Magnetic response of an organism and its lunar relationships. *Biol. Bull.,* **118** : 382–392.

Hartley, H. O., 1943. The maximum F-ratio as a short-cut test for heterogeneity of variance. *Biometrika,* **37** : 308–312.

Palmer, J. D., 1963. Organismic spatial orientation in very weak magnetic fields. *Nature,* **198** : 1061–1062.

SERUM ANTIBODY SYNTHESIS IN LARVAE OF THE BULLFROG, RANA CATESBEIANA[1]

E. L. COOPER,[2] W. PINKERTON AND W. H. HILDEMANN

Department of Medical Microbiology and Immunology, School of Medicine, University of California, Los Angeles 24, California

Much evidence now indicates that poikilothermic vertebrates are excellent forms for studies of developmental and phylogenetic aspects of immunogenetics (see reviews by Hildemann, 1962a, 1962b; Hildemann and Cooper, 1963). From a developmental viewpoint, some of these studies have been concerned with the maturation of immunologic responsiveness, with a greater emphasis devoted to the capacity to react to skin allografts, the temporal appearance of blood cell types and, more recently, the role of the thymus gland in the ontogeny of the immune system.

Although ample data are accumulating dealing with the allograft reaction in adult cold-blooded vertebrates, the immunological competence of these species as antibody producers has been at issue mainly because of insufficient study (Hildemann, 1962b; Papermaster *et al.,* 1963). Even though some reports have indicated specific precipitin production by adult amphibians (Evans and Horton, 1961; Hildemann, 1962b; Austin and Nace, 1962), no such serum antibody production has been previously demonstrated in larval amphibians. The extent and rate of maturation of the isoimmune response capacity in young poikilotherms *vis-à-vis* birds and mammals have posed interesting phylogenetic questions. Precipitins to xenogenic antigens have been induced in high titer by immunization of adult fishes (Ridgeway, 1962; Clem and Sigel, 1963) and adult amphibians (Evans, 1963; Evans and Horton, 1961; Austin and Nace, 1962), but no comparable data are at hand concerning the capacities of juvenile or larval recipients. Accordingly, this report describes new findings—the production of precipitins to xenogeneic and allogeneic serum protein antigens by larvae of the American bullfrog, *Rana catesbeiana.* This report, however, deals with the effects of thymectomy on the production of precipitins to xenogeneic antigens. Studies are now in progress which are aimed at understanding the relationship between the amphibian thymus gland, other lymphoid organs and the maturation of cellular and humoral immunity.

MATERIALS AND METHODS

Thymectomy of bullfrog larvae. In anuran larvae, the thymus gland is a bilateral, white, compact organ situated in close proximity to the eye and ear.

---

[1] This investigation was supported in part by Research Grant CA 04027 from the National Cancer Institute and by GRSG Grant 1-GS-52 from the U. S. Public Health Service.

[2] Post Doctoral Fellow of the National Cancer Institute (CPD-14,125). Present address: Department of Anatomy, School of Medicine, University of California, Los Angeles 24, California.

Although amphibians supposedly have no discrete lymph nodes comparable to those of mammals, they do have "lymph glands" which are likewise bilateral and located in the branchial region; the role of this accessory lymphoid tissue is still conjectural. The thymus gland was excised from both sides of the head after anesthetizing the tadpoles in 0.6% saline containing 42 mg. tricaine methane sulfonate/L. Sharp needles and iridectomy scissors were best for making the initial cut in the side of the head, while watchmaker forceps were used for removing the gland once it had been exposed. After the excision, as a precautionary measure, tetracycline wound powder was sprayed into the wound, which was finally closed by means of a single cat-gut suture. All operations were performed with the aid of a stereomicroscope. The tadpoles were placed in 0.6% saline for about 6–12 hours before they were returned to pond water, and were always maintained at a temperature of 25 ± 0.5° C. throughout the experiments. Bullfrog larvae were collected in the late summer when they were approximately 3–4 months of age and at standard stage 25. In contrast to this condition during the first year, tadpoles rapidly grow and usually approach metamorphosis in the wild sometime during the second year.

Immunization with xenogeneic antigens. Larvae of approximately 3–4 months of age, that had been previously thymectomized bilaterally at standard stage 25, were given initially a 0.05-ml. subcutaneous injection of goldfish (*Carassius auratus* Linnaeus, 1758) serum emulsified with incomplete Freund's adjuvant. Whole serum, obtained from several adult fish by cardiac puncture, was pooled. Booster injections of the same amount of antigen alone were given intraperitoneally 10–17 days later. The interval between thymectomy and initial antigenic challenge was in all cases at least 60 days. Tadpoles were bled individually from the tail and each serum sample obtained 9–11 days after the booster was tested against four dilutions (1:4; 1:8; 1:16; 1:32) of the antigen by means of the agar gel diffusion technique of Ouchterlony. The tests, set up on microscope slides placed in humid Petri dishes, were kept at room temperature. Lines of precipitation were observed after 2–3 days in all positive tests. Since some of the tests were negative in the sham-thymectomized control group, it was concluded that naturally occurring precipitins were not present.

Immunization with allogeneic antigens. Tadpoles ranging from stages 26–29 were immunized with pooled serum obtained from several adult bullfrogs by cardiac puncture. A total of six injections, each of 0.05 ml. allogeneic serum, was given at intervals of 10–17 days. The first and fifth doses were emulsified in incomplete Freund's adjuvant and injected subcutaneously; the other injections of serum alone were given intraperitoneally. Serum samples were obtained 11 days after the last booster, and control serum from ten non-immunized larvae and adults, each tested individually, revealed no naturally occurring isoprecipitins. The remaining experimental analysis is identical to that described above when goldfish serum was used as the antigen. Methods for the successful laboratory maintenance of bullfrog larvae have been previously described (Hildemann and Haas, 1959).

Technique of bleeding. One convenient region for obtaining blood from bullfrog larvae at varying intervals was the fleshy portion of the tail just caudal to the abdomen. To prevent any excess moisture which might be mixed with the blood, the tadpoles were first held firmly and wiped dry completely with cotton on one side. After a short quick stab into the tail with a sterile lancet, blood was collected easily and quickly with capillary pipettes and placed into culture tubes (60 × 50 mm.). When the clot had completely retracted after approximately one hour at room temperature, the tubes were centrifuged at 1500 rpm for 10 minutes. The serum was then placed in sterile culture tubes, stoppered and stored at −20° C.

RESULTS

Three of the eight larvae immunized over a two-month period with allogeneic serum yielded serums that gave isoprecipitin bands (Fig. 1a). Thus, the existence of serum isoantigens or serum groups in this species is evident. The apparent lack of isoprecipitin production in some recipients is attributable either to insufficient immunization or to sharing of major antigens with donor serums. Inasmuch as serums obtained from these larvae prior to the last two injections gave negative results, it appears that prolonged immunization is necessary for substantial isoprecipitin production.

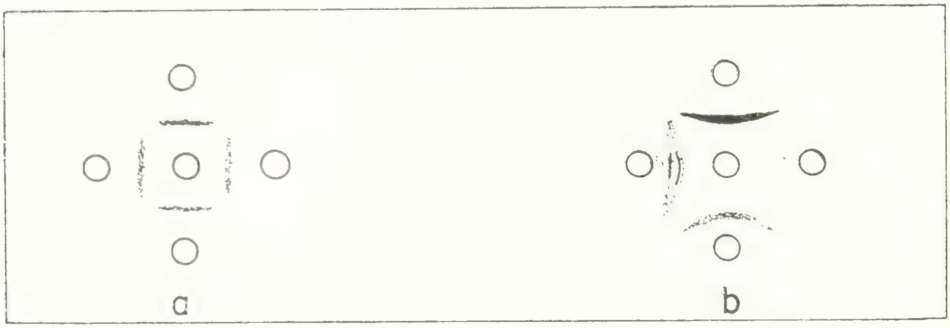


FIGURE 1 a, b. Characteristic precipitation patterns in double diffusion plates obtained from tadpoles immunized with adult serum (isoimmunization, Fig. 1 a), and adult goldfish serum (Fig. 1 b). Note differences in the precipitin bands between the two groups. Longer periods of immunization were necessary to produce isoprecipitins than precipitins to xenogeneic serum antigens. Six injections of antigen were required when using the allogeneic antigen. The first and fifth consisted of whole adult bullfrog serum emulsified in incomplete Freund's adjuvant. In the case of the xenogeneic group, only two injections were neecssary. The first consisted of antigen emulsified in Freund's adjuvant.

The data summarized in Table I support the thesis that bullfrog larvae are capable of synthesizing serum antibodies to xenogeneic antigens. However, the exact time at which the immune response toward diverse antigens matures has not yet been established.

In a group of 18 larvae that were sham-thymectomized and subsequently injected with goldfish serum antigen at stages 25–29, 10 showed strongly positive precipitin bands. Five serums reacted to produce weakly positive bands, while three were completely negative even after prolonged incubation. In a group of 13 larvae, however, which were thymectomized at stage 25–27, the precipitin band patterns were markedly different. In this group, only one showed a positive precipitin band, seven a weakly positive reaction, and five were negative. The

TABLE I

Precipitin reaction of bullfrog larval antiserum with goldfish serum antigen in Ouchterlony tests

No. of larvae immunized with goldfish serum	Standard stage at thymectomy	Group and standard stage at immunization and testing	Precipitin test
10		25-adult unoperated non-immunized	negative
18	25	25–29 sham-thymectomized	10 positive 5 weakly positive 3 negative
13	25	25–27 thymectomized	1 positive 7 weakly positive 5 negative

data suggest that thymectomy interfered with the humoral antibody response to serum protein antigens. However, the variation in antibody response between the two groups might to some extent depend upon differences in developmental age, *e.g.*, stage 25 versus stage 29 in relation to age at thymectomy. By comparing Figures 1a and b it is apparent that a shorter immunization schedule was sufficient to produce stronger precipitin bands to xenogeneic goldfish serum than to allogeneic serum. Because only small quantities of immune larval serum have been available, quantitative precipitin and immunoelectrophoretic analyses have yet to be performed.

DISCUSSION

Although precipitin production to soluble protein antigens by larval poikilotherms has been demonstrated in this investigation, the elucidation of the time in development when this capacity first appears remains problematical. The bullfrog has a prolonged larval period which is advantageous when dealing with developmental problems over a long period. Therefore, one important aspect of this study is to be able to show unequivocally the time of onset of the capacity to synthesize serum antibodies. It has already been demonstrated that larvae ranging in age from two months (stage 25) to two years vigorously reject skin allografts. However, newly hatched larvae at stage 24 and up to 36 days of age were sufficiently immature to become partially or completely tolerant toward allografts (Hildemann and Haas, 1959). The blood cell picture reveals that small lymphocytes begin to appear 40–45 days post-hatching during this transitional state of weak reactions to allografts (Hildemann and Haas, 1962). How the larvae resist environmental pathogens during the first six weeks post-hatching is conjectural. Abundant mucous secretions of the skin are no doubt important, while maternally-derived antibodies in the yolk may be essential until the larvae develop their own immunologically competent cells. It might be possible to correlate the disappearance of yolk, as the primary nutritive source, and the onset of feeding with the maturation of certain humoral responses after 15 days when leucocytes begin to appear.

The finding that thymectomized larvae appeared to show a weakened humoral antibody response to goldfish serum antigen is consistent with observations of mammals thymectomized early in life (Miller, 1962; Jankovic *et al.*, 1962). In avian vertebrates there exists a functional dichotomy between the thymus gland and the bursa of Fabricius. Here the thymus plays a dominant role in cellular immunity (Aspinall *et al.*, 1963) while the bursa, a cloacal lymphoid organ, is essential for humoral antibody responses (Graetzer *et al.*, 1963).

There is ample evidence now to promote vigorous debate concerning the mechanism by which the thymus mediates immunologic responsiveness (Levey *et al.*, 1963; Law *et al.*, 1964; Osoba and Miller, 1964), *i.e.*, whether it is exclusively cellular or humoral, or a combination of both mechanisms. The available evidence, supported by experiments using diffusion chambers, suggests that a humoral factor or hormone is active in juveniles and adults; however, the initial developmental acquisition of immunological competence and its subsequent maintenance may well depend upon systemic dissemination of thymocytes. The finding that bullfrog larvae thymectomized after about 50 days post-hatching show the usual acute rejection of allografts, but exhibit a weakened antibody response (Cooper *et al.*, 1963; Hildemann and Cooper, 1963) and conspicuous runting (Cooper, unpublished) supports the assumption that both cellular and humoral functions may be attributed to the thymus.

Perhaps the thymus in Amphibia continues to affect humoral antibody production and growth after the maturation of the immune response to skin allografts, but its influence is greatest on cellular immunity during the early larval period before general immunological competence is attained. In the case of skin allografts, the extent of the immune response is a function of the degree of immunogenetic unrelatedness between hosts and donors. Thus, graft survival in thymectomized mice is most prolonged in those strain combinations wherein weak histocompatibility barriers exist and least produced across strong H-2 differences (Martinez *et al.*, 1962). Although many "strong" histocompatibility alleles at various loci are evident in bullfrog populations, a high degree of immunologic specificity of allograft rejection in bullfrog larvae has also been demonstrated (Hildemann and Haas, 1961). With regard to serum antibody production, the immune response may also be substantially influenced by host genotype relative to different donor antigens, and by the schedule of immunization. Another factor which could affect the degree of immunologic impairment in thymectomized larvae is a variable regeneration of thymic rudiments and differences in maturation rate relative to age. In another study (Cooper and Hildemann, in preparation) concerned with the relationship between thymectomy and skin allograft survival, all larvae were biopsied at the end of the experimental period to determine the condition of the thymus, since it was assumed that all tissue had been removed initially. A detailed study is necessary to determine with certainty whether the thymus in the bullfrog is capable of undergoing regeneration.

In contrasting the production of precipitins to xenogeneic antigens (goldfish serum) with precipitins to allogeneic serum (*Rana catesbeiana* Shaw 1802), it seems appropriate to consider several factors. With regard to the production of antibodies against xenogeneic antigens, the class- and species-specific differences in the composition of serum proteins existing between the donor and recipients are sufficiently numerous to readily assure the induction of antibody production. By contrast, the fewer individual differences existing between serum proteins within a species may also represent minor alterations in molecular structure that are effectively less antigenic or foreign—thus the longer period of immunization required for the production of isoantibodies. In the bullfrog subpopulation presently studied, a number of potential isoantigens (allotypic specificities) may be shared by all of the individuals tested; in other words, only a few genes determining serum protein specificity may be segregating in this population. Dray and Young (1958), in their successful demonstration of induced isoprecipitins in certain rabbits, focused attention on the importance of immunization schedules. With appropriate techniques, isoantibodies are capable of detecting not only wider antigenic differences between the several globulin fractions of serum, but also the allotypic specificities within the gamma fraction alone.

Although the present investigation has thus far been primarily concerned with the maturation of immunological competence, ancillary findings involve immunogenetic and comparative immunological concepts as well. In addition, a study of this kind devoted to an understanding of the ontogeny of the immune system in an amphibian such as the bullfrog will help to clarify concepts regarding the evolution of the immune response.

We thank Mr. James F. Godfrey for preparation of the photographs.

Summary

1. Larvae of the American bullfrog, *Rana catesbeiana*, were thymectomized and sham-thymectomized at stages 25–29, representing an age range of about 60–200 days. After immunization at 25° ± 0.5° C. with goldfish serum proteins as antigens, specific precipitating antibodies were found in the majority of the sham-operated controls. In contrast, nearly all of the thymectomized group showed a markedly weakened response to goldfish serum antigens.

2. Lines of isoprecipitation were obtained in Ouchterlony tests with serums from some larvae immunized with isoantigens (whole adult serum). A longer period of immunization is apparently required to produce precipitins to serum isoantigens than to comparable xenogeneic antigens. To our knowledge, this is the first finding of serum isoantigens or allotypes and induced isoprecipitins in any poikilothermic vertebrate.

LITERATURE CITED

Aspinall, R. L., R. K. Meyer, M. A. Graetzer and H. R. Wolfe, 1963. Effect of thymectomy and bursectomy on the survival of skin homografts in chickens. *J. Immunol.*, **90**: 872–877.

Austin, L. G., and G. W. Nace, 1962. Precipitating antibody production in *Rana pipiens*. *Bact. Proc.*, p. 74.

Clem, L. W., and M. M. Sigel, 1963. Comparative immunochemical and immunological reactions in marine fishes with soluble, viral and bacterial antigens. *Fed. Proc.*, **22**: 1138–1144.

Cooper, E. L., W. H. Hildemann and W. Pinkerton, 1963. Serum antibody synthesis and skin homograft survival in larvae of the bullfrog *Rana catesbeiana:* Role of the thymus gland. *Immunogenetics Letter*, **3**: 62–67.

Dray, S., and G. O. Young, 1958. Differences in the antigenic components of sera of individual rabbits as shown by induced isoprecipitins. *J. Immunol.*, **81**: 142–149.

Evans, E. E., and S. L. Horton, 1961. Synthesis and electrophoretic distribution of antibodies in the amphibian, *Bufo marinus*. *Proc. Soc. Exp. Biol. Med.*, **107**: 71–74.

Evans, E. E., 1963. Antibody response in amphibia and reptilia. *Fed. Proc.*, **22**: 1132–1137.

Graetzer, M. A., H. R. Wolfe, R. L. Aspinall and R. K. Meyer, 1963. Effect of thymectomy and bursectomy on precipitin and natural hemagglutinin production in the chicken. *J. Immunol.*, **90**: 878–887.

Hildemann, W. H., 1962a. Immunogenetic studies of poikilothermic animals. *Amer. Nat.*, **96**: 195–204.

Hildemann, W. H., 1962b. Immunogenetic studies of amphibians and reptiles. *Ann. N. Y. Acad. Sci.*, **97**: 139–152.

Hildemann, W. H., and E. L. Cooper, 1963. Immunogenesis of homograft reactions in fishes and amphibians. *Fed. Proc.*, **22**: 1145–1151.

Hildemann, W. H., and R. Haas, 1959. Homotransplantation immunity and tolerance in the bullfrog. *J. Immunol.*, **83**: 478–485.

Hildemann, W. H., and R. Haas, 1961. Histocompatibility genetics of bullfrog populations. *Evolution*, **15**: 267–271.

Hildemann, W. H., and R. Haas, 1962. Developmental changes in leukocytes in relation to immunological maturity. *In:* Symposium on Mechanisms of Immunological Tolerance. Prague: Czechoslovakia Acad. Sci., p. 35.

Jankovic, B. D., B. H. Waksman and B. G. Arnason, 1962. Role of the thymus in immune reactions in rats. I. The immunologic response to bovine serum albumin (antibody formation, arthus reactivity, and delayed hypersensitivity) in rats thymectomized or splenectomized at various times after birth. *J. Exp. Med.*, **116**: 159–176.

Law, L. W., N. Trainin, R. H. Levey and W. F. Barth, 1964. Humoral thymic factor in mice: Further evidence. *Science*, **143**: 1049–1051.

Levey, R. H., N. Trainin, L. W. Law, P. H. Black and W. P. Rowe, 1963. Lymphocytic choriomeningitis infection in neonatally thymectomized mice bearing diffusion chambers containing thymus. *Science*, **142**: 483–485.

Martinez, C., J. Kersey, B. W. Papermaster and R. A. Good, 1962. Skin homograft survival in thymectomized mice. *Proc. Soc. Exp. Biol. Med.*, **109**: 193–196.

Miller, J. F. A. P., 1962. Effect of neonatal thymectomy on the immunological responsiveness of the mouse. *Proc. Roy. Soc. London, Ser. B*, **156**: 415–428.

Osoba, D., and J. F. A. P. Miller, 1964. The lymphoid tissues and immune responses of neonatally thymectomized mice bearing thymus tissue in Millipore diffusion chambers. *J. Exp. Med.*, **119**: 177–194.

Papermaster, B. W., R. M. Condie, J. K. Finstad, R. A. Good and A. E. Gabrielson, 1963. Phylogenetic development of adaptive immunity. *Fed. Proc.*, **22**: 1152–1155.

Ridgeway, G. J., 1962. The application of some special immunological methods to marine population problems. *Amer. Nat.*, **96**: 219–224.

MORPHOLOGICAL COLOR CHANGE IN THE FIDDLER CRAB, UCA PUGNAX (S. I. SMITH)[1, 2]

JONATHAN P. GREEN[3]

Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and the Department of Zoology, University of Minnesota, Minneapolis, Minnesota 55455

Morphological color changes have been defined (Green, 1963) as enduring modifications of the pigmentary system resulting from production or destruction of pigment and/or chromatophores. Morphological color changes can occur in a variety of ways: (1) by an increase (decrease) in the number of chromatophores per unit area of body surface (Bowman, 1942; Green, 1963, 1964); (2) by an increase (decrease) in the pigment concentration (Brown, 1934; Green, 1963); or (3) by a combination of the above.

Babak (1912) postulated a relationship between physiological and morphological color changes based on the state of dispersion of pigment within the chromatophores. This relationship, subsequently called Babak's Law, stated that the maintenance of a pigment in a concentrated state within the chromatophore was correlated with a reduction in the quantity of that pigment. Conversely, pigment dispersion was associated with pigment production.

The present study deals with part of the pigmentary system of the fiddler crab, *Uca pugnax,* and with its capacity to undergo morphological color changes.

MATERIALS AND METHODS

Crabs were collected on the salt flats at Sippewissett Beach, Cape Cod, Massachusetts. The animals were brought to the laboratory and placed in a large tank with continuously running sea water covering the bottom to a depth of $\frac{1}{2}$ inch. Crabs were selected at random from this group (usually about 100 animals) for the various experiments. In all cases males were used and the great chela was removed by forcing the animal to autotomize the appendage. This procedure reduced the hemocoel space and thereby increased the efficiency of incorporation of radioactive precursors.

Tyrosine-C^{14} was injected into the crabs as a precursor of the black pigment. In one series of experiments dihydroxyphenylalanine-C^{14} (DOPA) was used in place of tyrosine. All injections were made into the hemocoel through the arthrodial membrane between the coxopodite and the ischiopodite of the fourth walking

---

[1] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from the Department of Zoology, University of Minnesota, Minneapolis, Minnesota 55455.

[2] Supported by a predoctoral research fellowship from the National Institutes of Health, U.S.P.H.S. (G-9853-C1; GPM 9853-C2).

[3] Present address: Department of Biology, Brown University, Providence, Rhode Island 02912.

leg. After the injection of the tyrosine the crabs were placed on various backgrounds. The animals were unfed during the experimental period (which amounted to at most 20 days for any one animal). After the appropriate time on the background the approximate area of the carapace was determined. The carapace was then dissected from the animal and freed of adhering tissues. This preparation was composed of the exoskeleton lined with epidermis in which the pigment cells lie. The carapace was cut into four pieces and dropped into a stainless steel centrifuge tube. Ten ml. of distilled water were added. The material was sonicated in a M. S. E. sonicator with a $\frac{3}{4}$-inch probe for 2.5 minutes at 20 kc. An additional 10 ml. of water were added after the suspension was decanted and the process was repeated. All of the pigment granules were removed from the hypodermis after 2.5 minutes of sonication; the additional period of sonication served as a rinse. The resulting 20 ml. of dark colored fluid were centrifuged (2100 *g*) in an International centrifuge for 20 minutes. A black pellet was found in the tubes after centrifugation. Subsequent re-suspension and re-centrifugation with 5 ml. of distilled water, for a total of three washings (in all), was sufficient to remove any extraneous material. The pigment was then suspended in a minimum quantity of water (less than 1 ml.) and pipetted onto aluminum planchets ringed with wax. The suspension was dried over a hot plate and the radioactivity of the pigment measured with a thin-window Geiger tube.

In the following discussion the values reported are the mean number of counts per minute per square centimeter of carapace ± the standard error of the mean. In all cases the count has been corrected for background radiation. The samples were not corrected for self-adsorption which may be significant; therefore these measurements represent relative and minimal values for incorporation.

RESULTS

Identification of the pigment

The fiddler crab, *Uca pugnax*, has black, red, white and yellow chromatophores. The black and white chromatophores play the major role in determining the shade of the animal. The dark pigment within the black chromatophores has not been previously characterized. In the present work it is identified partially on the basis of its solubility and reaction to strong oxidizing agents. The black pigment was treated with 1 N HCl, 95% and 100% ethyl alcohol, 95% and 100% methyl alcohol, acidic ethyl and methyl alcohols (HCl 5% v/v), acetone, ethylene chlorhydrin, and 0.5 N NaOH. The pigment was soluble only in the last two of the listed solvents. The extracted and washed pigment granules were bleached by 10% hydrogen peroxide in 24 hours at 5° C.

Incorporation studies

Injected tyrosine-C^{14} or DOPA-C^{14} was incorporated into the black pigment *in vivo*. The radioactive label was not removed by exhaustive washing of the extracted pigment with distilled water, 95% or 100% ethyl alcohol. Precipitation of L-tyrosine-1-C^{14}-labeled pigment from 0.5 N NaOH solutions with 0.1 N HCl yielded precipitates with levels of radioactivity not different from that of the untreated pigment.

As a further check on the incorporation of C^{14} into the black pigment, crabs were injected with 0.5 μC. of L-valine-1-C^{14} and with 0.5 μC. of glycine-1-C^{14}. After 24 hours on neutral background with natural lighting no significant levels of radioactivity could be found in the pigment extracted from crabs injected with either amino acid.

Concentration curve

Forty crabs, in groups of four, received varying amounts of DL-tyrosine-2-C^{14}. After 24 hours on a neutral background with natural lighting, the area of the carapace was determined and the pigment extracted from the epidermis. Interpolation from a standard curve relating the area of the carapace to the wet weight of dechelate male crabs gave the total body weight. On this basis the μg. of tyrosine-C^{14} injected per gram wet weight of the crabs was calculated. A regression analysis of μg. tyrosine injected per gram wet weight against counts per minute per square centimeter of carapace of extracted pigment was undertaken.

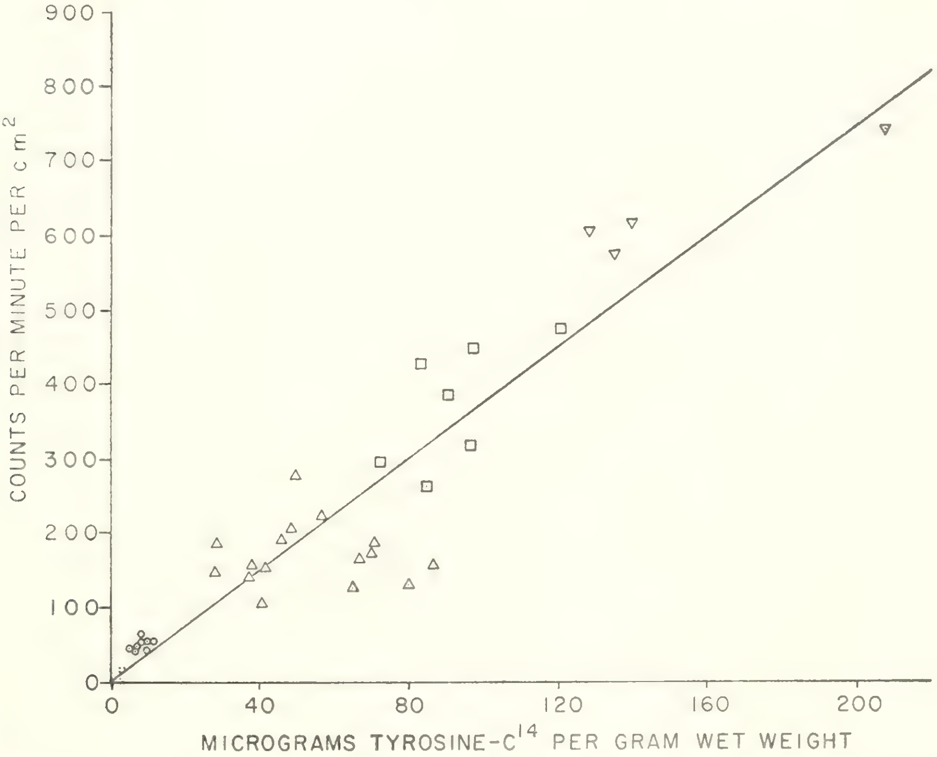


FIGURE 1. The relationship between the counts per minute per square centimeter of carapace of the extracted pigment and micrograms DL-tyrosine-2-C^{14} injected per gram wet weight. The linear regression line has been calculated and the $P = 0.95$ confidence interval of the slope is given. $Y = 3.72X + 5.35$. $3.27 \leq b \leq 4.17$. $P < 0.001$. Solid dots = 0.01 μC.; ⊙ = 0.10 μC.; △ = 0.50 μC.; ⊡ = 1.00 μC.; ▽ = 2.00 μC.: microcuries injected per animal.

The relationship between μg. tyrosine injected per gram wet weight and the radioactivity of the extracted pigment is linear within the experimental range, 0.88–208.00 μg. per gram (0.01–2.00 μC. tyrosine-C^{14} injected per animal). (Fig. 1). From this series of experiments it was determined that 1.05% of the injected tyrosine-C^{14} was recovered as labeled pigment.

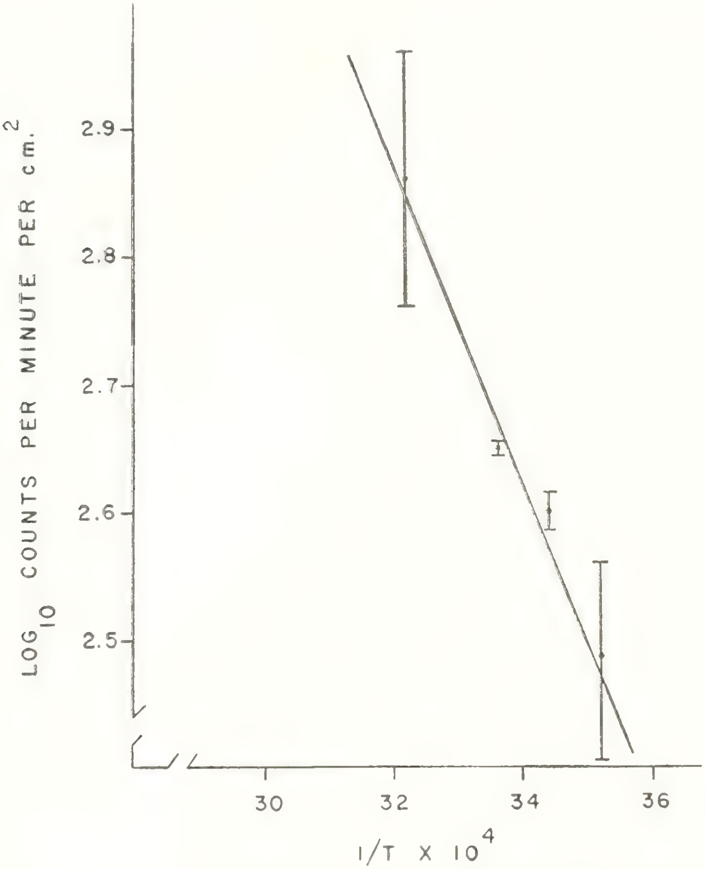


FIGURE 2. Arrhenius plot of data on incorporation of DL-tyrosine-2-C^{14} into the black pigment of the fiddler crab. The linear regression line has been calculated and the $P = 0.95$ confidence interval of the slope is given. $Y = -1.257X + 6.90$. $-0.657 \leq b \leq -1.857$. $P < 0.001$.

Temperature curve

Crabs were maintained at various temperatures for 24 hours after the injection of 1.0 μC. per crab of DL-tyrosine-2-C^{14}. The pigment was extracted and the counts per minute per $cm.^2$ of carapace were determined. The regression analysis indicated that a significant linear relationship existed between the logarithm of the counts per minute per $cm.^2$ of carapace and the reciprocal of the absolute temperature (Fig. 2). The Q_{10} (11–37° C.) is 1.42. From the slope of the Arrhenius plot of the data, the activation energy (μ) for the incorporation of tyrosine-C^{14} into the black pigment has been calculated to be 5757 calories.

DL-3(3,4-dihydroxyphenyl)alanine-2-C^{14} (DOPA) as a precursor of the black pigment

Fourteen crabs received 0.5 μC. DOPA-C^{14}. The animals were maintained under conditions of neutral background and constant illumination for a period of 24 hours. After this time the animals were weighed and the pigment was extracted from the epidermis. A mean value of 111.9 ± 11.6 counts per minute per cm.^{2} was obtained. One per cent of the injected DOPA-C^{14} was recovered as labeled pigment.

Incorporation studies with normal animals

Pigment dispersion within the chromatophores of fiddler crabs fluctuates periodically, both in the normal environment and under constant conditions of illumination and temperature in the laboratory (Megusar, 1912; Abramowitz, 1937; Brown and Webb, 1948; Stephens, 1957). The migration of pigment into pre-established chromatophore channels is controlled primarily by various hormones or chromatophorotropins. Some of these chromatophorotropins appear to be released in a cyclic manner so that the black chromatophore pigment is expanded during the daylight hours and contracted at night.

TABLE I

Comparison between radioactivity levels of pigment extracted from crabs injected with tyrosine-C^{14} at noon and at midnight

Treatment	N	Mean ± S. E.	Mean chromatophore stage				
			Hour				
			0	1	2	3	4
Injected at 1200 hours, extracted at 1600 hours	4*	15.70 ± 1.44	5.0	4.8	5.0	4.6	4.4
Injected at 0000 hours, extracted at 0400 hours	4*	15.52 ± 0.42	1.2	1.2	1.0	1.0	1.0
Injected at 1200 hours, extracted at 1600 hours	8**	255.75 ± 30.49	5.0	4.8	4.3	4.3	3.9
Injected at 0000 hours, extracted at 0400 hours	8**	296.75 ± 53.89	1.6	2.5	1.6	1.3	1.2

* 0.1 μC. L-tyrosine-1-C^{14} per animal.
** 1.0 μC. DL-tyrosine-2-C^{14} per animal.

In an attempt to correlate synthesis of the black pigment with the state of pigment dispersion within the chromatophore (Babak's hypothesis) the following experiments were designed. Crabs were injected with L-tyrosine-1-C^{14} at noon and at midnight. Four hours later the pigment was extracted. The experiment was repeated with injections of DL-tyrosine-2-C^{14}.

The heavy exoskeleton covering the epidermis of the carapace prevented detailed observation of the underlying chromatophores. Therefore, the state of dispersion of the pigment in pereiopod chromatophores was utilized as a general index of chromatophore activity. It is possible that the pigment of the carapace chromatophores responds differently than does the pigment of pereiopod chromatophores. No information is available concerning the exact relation between the chromatophore pigments of these areas.

During the four-hour experimental period black chromatophores were staged according to the scheme of Hogben and Slome (1931). From 0000-0400 hours the pigment within the black chromatophores is contracted (stage 1) and during the period 1200–1600 hours it is dispersed (stage 5). Subsequent extraction of the pigment and measurement of its radioactivity levels revealed that there was no difference between the animals injected at midnight and those injected at noon (Table I).

Table II

Comparison between radioactivity levels of pigment extracted from normal and destalked crabs receiving eye-stalk extracts

Treatment	N	Mean ± S. E.	Chromatophore stage
Normal animals			
All animals received 0.5 μC. L-tyrosine-1-C^{14} every four hours for a total of 24 hours			
1. Plus 0.05 ml. of sea water every four hours for a total of 24 hours	4	239.25 ± 10.55	Varies from ~5 at 1200 hours to ~1 at 0000 hours
2. Plus 0.05 ml. eye-stalk extract containing the equivalent of ½ eye-stalk, every four hours for a total of 24 hours	4	262.50 ± 23.30	~5
Destalked animals			
All animals received 0.5 μC. L-tyrosine-1-C^{14} every four hours for a total of 24 hours			
1. Plus 0.05 ml. sea water every four hours for a total of 24 hours	4	381.50 ± 27.75	~1
2. Plus 0.05 ml. eye-stalk extract containing the equivalent of ½ eye-stalk, every 4 hours for a total of 24 hours	4	339.25 ± 25.10	~5

Incorporation studies with eye-stalkless animals (destalked animals)

In the following experiments the effect of an injection of eye-stalk extract on the synthesis of the black pigment was studied. Sixteen crabs were selected, eight normal crabs and eight destalked crabs. The pigment of the black chromatophores of destalked crabs assumes and maintains the contracted position (stage 1), mimicking the condition normally seen only at night (Megusar, 1912; Carlson, 1935). Each crab received 3 μC. of L-tyrosine-1-C^{14} in 0.5-μC. injections every four hours for 24 hours. One-half the animals, four normal and four destalked, also received 0.05 ml. of an eye-stalk extract containing the equivalent of one-half eye-stalk. The eye stalk extract was made by grinding eye-stalks with sterile sand and artificial sea water in a mortar. The resulting brei was centrifuged and subsequently heated in a boiling-water bath for five minutes. The material was re-centrifuged and the supernatant was brought to such a volume that 0.05 ml. contained the equivalent of one-half eye-stalk. This technique for the extraction of chromatophorotropin is a variant of that used by Brown (1940). Injection of the equivalent of one-half eye-stalk led to an expansion of the pigment within the black chromatophores (see, also, Sandeen, 1950; Fingerman *et al.*, 1964) and this effect persisted for approximately four hours. Therefore, with the injection of one-half eye-stalk equivalents every four hours the black chromatophore pigment was induced to remain expanded for the entire experimental period of 24 hours. The results of these experiments indicated that the injection of eye-stalk extract had no significant effect on the level of radioactivity of the extracted pigment from either normal or destalked crabs (Table II). However, there was a difference in the level of radioactivity of the extracted pigment between normal and destalked crabs. The level of radioactivity of the destalked crabs' pigment was significantly higher than that of the normal crabs.

TABLE III

Comparison between radioactivity levels of pigment extracted from destalked crabs receiving injections of sea water, eye-stalk extract and/or phenylthiourea

Treatment	N	Mean ± S. E.	Chromatophore stage					
			Minutes after injection					
			0	15	30	45	75	135
All animals received 0.1 μC. L-tyrosine-1-C^{14} plus:								
1. 0.1 ml. Sea Water	4	93.62 ± 4.7	1.4	1.4	1.8	1.2	1.2	1.2
2. ½ eye-stalk	4	97.42 ± 4.5	1.2	4.0	4.0	4.4	4.4	4.4
3. 1 mg. PTU	4	31.50 ± 7.8	1.4	1.8	1.8	2.2	1.2	1.2
4. ½ eye-stalk + 1 mg. PTU	4	24.60 ± 5.3	1.2	3.6	4.2	4.4	4.8	4.4

Inhibition studies

The role of chromatophorotropins and the effect of phenylthiourea (PTU) on the formation of the black pigment were investigated. PTU inhibits the tyrosine-tyrosinase reaction by binding the copper prosthetic group of tyrosinase (Lerner and Fitzpatrick, 1950). The direct action of PTU on the black chromatophores was tested as follows: 15 normal crabs were injected with 1 mg. PTU in sea water (0.1 ml.) each at 1200 hours, 15 control crabs received 0.1 ml. sea water at the same time. The black chromatophores were staged every 15 minutes for one hour and hourly thereafter for a total of four hours. There was no difference in chromatophore stage between the two groups. With this assurance that PTU had no effect on chromatophore stage, 16 destalked crabs received 0.1 μC. of L-tyrosine-1-C^{14} each. These crabs were also treated with eye-stalk extract and/or PTU. After four hours the pigment was extracted and the radioactivity measured (Table III).

Another experiment was performed using eight normal and eight destalked crabs with injections of PTU every six hours for a total of 24 hours. The animals were maintained on a neutral background with natural lighting (Table IV).

From the preceding results it appears that a mechanism involved in the control of pigment dispersion cannot be concerned with pigment elaboration *per se.*

TABLE IV

Comparison between radioactivity levels of pigment extracted from normal and destalked crabs receiving injections of sea water or phenylthiourea

Treatment	N	Mean ± S. E.	Chromatophore stage
All animals received 1.0 μC. of DL-tyrosine-2-C^{14} at the start of the experiment			
Normal animals			
1. 0.05 ml. sea water every 6 hours	4	313.50 ± 25.6	Varies from $\sim$5 at 1200 hours to $\sim$1 at 0000 hours
2. 1 mg. PTU every 6 hours	4	170.25 ± 11.0	Varies from $\sim$5 at 1200 hours to $\sim$1 at 0000 hours
Destalked animals			
3. 0.05 ml. sea water every 6 hours	4	495.50 ± 29.6	$\sim$1
4. 1 mg. PTU every 6 hours	4	173.25 ± 17.9	$\sim$1

Long-term studies

Twenty crabs each received 0.5 μC. of L-tyrosine-1-C^{14}. The crabs were maintained in glass jars with a small amount of sea water which was changed daily. The jars were kept on a neutral background with natural lighting. One, two, four, eight, and sixteen days after injection, pigment was extracted from members of this group and the radioactivity measured. In a parallel experiment, destalked crabs were similarly treated, although data were obtained only through eight days (Fig. 3). Values for the level of radioactivity of the extracted pigment from destalked crabs were 72% higher than those from normal animals. However, there is no significant change in the level of radioactivity over an 8-day period (destalked crabs) or over a 16-day period (normal crabs) compared to that which obtained after one day.

Background effects

1. Pigment formation

The effect of background shade on pigment formation was investigated. Eight crabs each received 0.5 μC. of L-tyrosine-1-C^{14}. They were placed individually in fingerbowls lined with either white or black cloth moistened with sea water. The crabs were kept in these bowls under conditions of constant illumination. After four hours the crabs were removed, the area of the carapace measured, the pigment extracted, and the radioactivity determined. No significant difference was found in the level of radioactivity of the pigment extracted from crabs maintained on either of the two backgrounds. There was also no significant difference between the black or the white groups and the control animals from Figure 1 (0.5 μC. group).

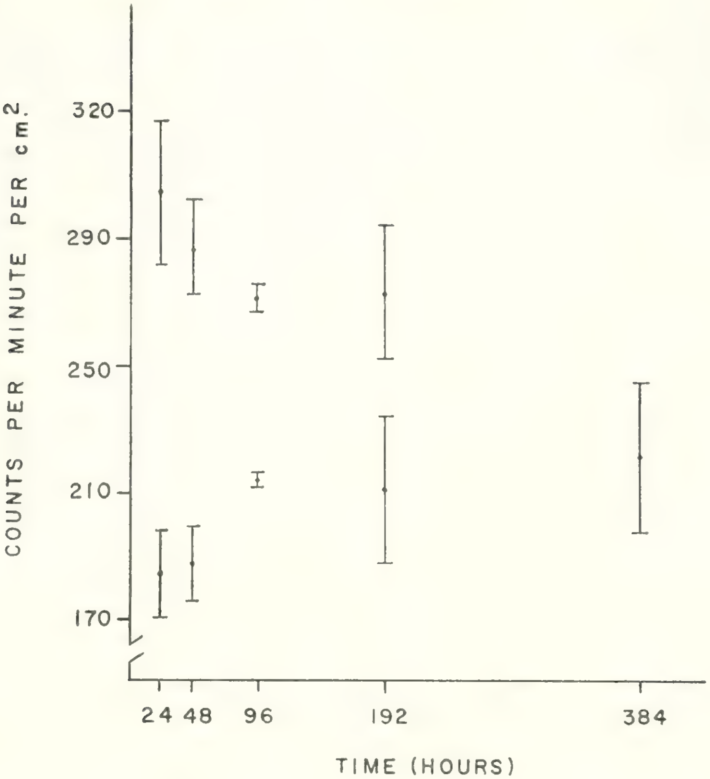


FIGURE 3. The relationship between the counts per minute per square centimeter of carapace of the extracted pigment and length of time spent on neutral background (natural illumination). Upper points for destalked animals, lower points for normal animals. The $P = 0.95$ confidence interval of the slope of the regression line is given. Destalked animals. $-0.50 \leq b \leq 0.02$. Normal animals. $-0.02 \leq b \leq 0.22$.

The effect of background shade on the maintenance of a level of radioactivity in pre-labeled pigment was investigated. Fifteen crabs received 0.1 μC. of L-tyrosine-1-C^{14} each. The crabs were segregated in groups of five and kept in glass jars on a neutral background with natural lighting. Twenty-four hours later four crabs

TABLE V

Comparisons between radioactivity levels of pigment extracted from pre-labeled crabs maintained for 72 hours on white or black backgrounds

Treatment	N	Mean ± S. E.
All animals received 0.1 μC. L-tyrosine-1-C^{14}. Maintained for 24 hours on a neutral background		
Control—24 hours	4	22.2 ± 1.8
Black background—constant illumination—72 hours	4	21.2 ± 3.2
White background—constant illumination—72 hours	4	1.3 ± 0.5

were randomly selected and the pigment extracted. The remaining two groups of five animals were placed on either a white or a black background under conditions of constant illumination. Seventy-two hours later the animals were removed and the radioactivity of the pigment measured (Table V). No significant differences exist between animals maintained on a black background for 72 hours and the control animals (sacrificed after 24 hours on a neutral background). Highly significant differences exist between control animals and those maintained on a white background, and between animals on a white background and on a black background.

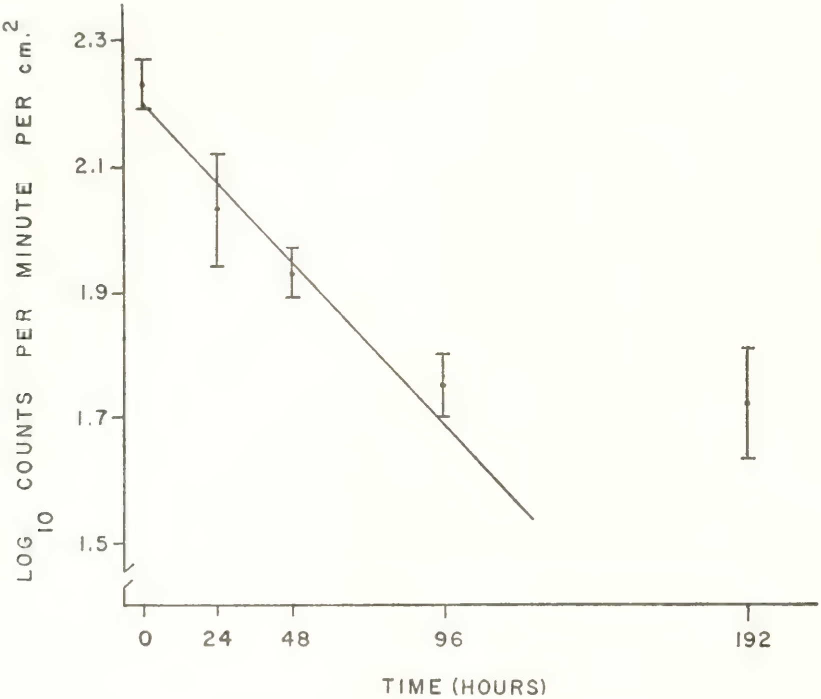


FIGURE 4. The relationship between the logarithm$_{10}$ of the counts per minute per square centimeter of carapace of the extracted pigment and length of time spent on a white brackground (constant illumination). A linear regression line has been calculated for the first 96 hours spent on a white background. The $P = 0.95$ confidence interval of the slope is given. Y $- 0.0053X + 2.208$. $-0.0066 \leq b \leq -0.0039$. $P < 0.001$.

Sixteen crabs received 0.5 μC. L-tyrosine-1-C^{14} each and were placed in groups of four on a neutral background with natural lighting. Twenty-four hours later the crabs were placed on a white background with constant illumination. Twenty-four, 48, 96, and 192 hours later four crabs were removed and the pigment extracted (Fig. 4). The 0.5-μC. animals from Figure 1 served as a control group.

A regression analysis has been carried out, using the logarithm of the counts per minute per cm.^{2} of carapace *versus* length of time on white background (Fig. 4). The greatest decrease (to a level 63.3% of the original level) occurred within

24 hours of placing the animals on a white background. The destruction appeared to occur exponentially for at least the first 96 hours. Fifty per cent of the label was lost from the pigment within 48 hours of placing the crabs on a white background. In a comparable experiment when pre-labeled animals remained on the white or black background for only four hours, no significant differences appeared between these four-hour animals and control animals.

Five crabs were injected with 0.5 μC. of DL-tyrosine-2-C^{14} and placed on a neutral background for 24 hours. They were then transferred to a box which illuminated the crabs from below while presenting a black background on all sides and from above. After 48 hours the crabs were removed and the pigment extracted. The level of radioactivity of the pigment extracted from these crabs was not significantly different from that which was obtained from animals maintained on a neutral background for 24 hours.

Discussion

Nature of the Uca black pigment

The black chromatophores of the fiddler crab, *Uca pugnax,* contain a granular pigment which is easily extracted. This pigment has been called a melanin (witness the name melanophore given to these chromatophores), although definitive proof for the nature of the pigment has never been given. In the present work the *Uca* black chromatophore pigment has been identified as a melanin on the basis of (1) solubility and bleaching reactions; (2) incorporation of tyrosine and DOPA into the pigment; and (3) inhibition of this incorporation by phenylthiourea, a known inhibitor of melanin formation.

Melanin pigments occur as intracellular granules which are relatively insoluble, capable of reducing silver nitrate, and are bleached by strong oxidizing agents (Lison, 1936; Pearse, 1961; Seiji *et al.*, 1963). Ommochrome pigments occur in the integument and eyes of arthropods and have often been confused with the melanins (Needham and Brunet, 1957; Forrest, 1959). Ommochromes can be distinguished from melanins on the basis of their solubility in acidic ethanol (HCl 5% v/v) and in acidic methanol (HCl 5% v/v) (Needham and Brunet, 1957). Because of the solubility properties of the *Uca* integumental black pigment it is highly improbable that this pigment is an ommochrome. The *Uca* black pigment is soluble in 0.5 N NaOH and in ethylene chlorhydrin, as are other melanins (Gortner, 1910; Lea, 1945).

According to the classical scheme for melanin formation in vertebrates, the amino acid tyrosine is enzymatically oxidized to 3,4-dihydroxyphenyl-L-alanine (DOPA). With the help of the same enzyme (tyrosinase) DOPA is oxidized to DOPA-quinone. Subsequently two other quinones are formed which may react with available proteins. The quinone-protein complex then polymerizes to form a black melanoprotein (Mason, 1948; Lerner and Case, 1959; Seiji *et al.*, 1963). In the present work, the incorporation of tyrosine-C^{14} and DOPA-C^{14} into the *Uca* black pigment further indicates that this pigment is a melanin.

Inhibition of the tyrosine-tyrosinase and DOPA-tyrosinase reactions usually occurs by the elimination of copper ions which are necessary for tyrosinase activity (Lerner and Fitzpatrick, 1950; Attie and Khafif, 1964). The majority of known inhibitor substances contain thiourea groups (Attie and Khafif, 1964). In the present study, phenylthiourea was found to inhibit strongly the incorporation of tyrosine-C^{14} into the melanin. This provides additional evidence for the melanin nature of the *Uca* black pigment.

Characteristics of the incorporation of tyrosine into Uca melanin

The concentration curve shown in Figure 1 indicates that incorporation of tyrosine-C^{14} into the melanin is linearly related to micrograms tyrosine injected. The time period in this experiment was 24 hours; however, comparable levels of radioactivity were found in pigment extracted from crabs which had been injected only four hours before extraction. Therefore, it appears that melanin synthesis occurs rapidly under normal conditions and may be related to the influx of free tyrosine.

The incorporation of tyrosine-C^{14} into the melanin is temperature-dependent, as can be seen from Figure 2. The energy of activation (μ), as calculated from the Arrhenius plot of the data, is 5757 calories. This figure is comparable to values in the literature for the *in vitro* reaction; Gould (1939) has reported a value for μ of 2700 calories for mealworm tyrosinase with catechol as a substrate. Lerner *et al.* (1949) report a Q_{10} (27–37° C.) for mammalian tyrosinase of 1.2, which is equivalent to $\mu = 3577$ calories, with either tyrosine or DOPA as a substrate.

In an attempt to follow the time course of the disappearance of the labeled melanin, crabs were injected with tyrosine-C^{14} and random samples were drawn from the population two, four, eight, and sixteen days after injection. The animals were kept on a neutral background with natural lighting. The level of radioactivity of the extracted pigment did not vary over the experimental period, indicating the stability of the melanin under these conditions.

Eye-stalkless crabs under similar conditions have a level of incorporation approximately 70% higher than do normal animals. They also retain the same level of radioactivity of the extracted pigment over the experimental period (8 days). The metabolism of destalked *versus* normal crustaceans has been investigated by Edwards (1952), Schwabe *et al.* (1952), Kincaid and Scheer (1952), Bliss (1953), Neiland and Scheer (1953), Guyselman (1953), and Carlisle and Knowles (1959). Two of the above papers offer a suggestion as to why the level of radioactivity of extracted pigment should be higher in destalked crabs. Schwabe *et al.* (1952) observed that a hormonal principle from the eye-stalk of *Panulirus japonicus* restrains the growth of the epidermal layer of the integument and with the removal of the eye-stalks, growth of the epidermis occurs. Neiland and Scheer (1953) extended these observations to *Hemigrapsus nudus* and found that normal catabolism of proteins is greatly increased by eye-stalk removal. However, anabolic processes involved in the molt, and especially in the formation of epidermal tissue, are accelerated. In the light of these findings, ablation of the eye-stalks of *Uca* probably leads to increased activity in the epidermis which is reflected by the incorporation of higher levels of tyrosine into melanin.

The responses of the melanophores of the fiddler crab to varying conditions of background shade, illumination, and state of pigment dispersion have been investigated by measuring the incorporation of a radioactive precursor into the pigment. Experiments were performed on crabs whose melanin pigment was in various stages of dispersion, both naturally-occurring and artificially-induced. The normal diurnal cycle of alternate pigment expansion (1200 hours) and contraction (0000 hours) offered the possibility of testing animals during normally-occurring states of pigment dispersion.

In another experiment injections of eye-stalk extracts every four hours kept the pigment expanded even during the time (0000 hours) when it would normally be contracted. Destalked animals in which the pigment was maximally contracted were also utilized. Injection of eye-stalk extract served to expand the pigment of destalked animals, and repetitive injections maintained it in the expanded state for 24 hours.

The level of incorporation of tyrosine-C^{14} into the melanin of destalked crabs was in all cases considerably higher than that in normal crabs. If this increase is neglected and an attempt is made to correlate the formation of melanin with the degree of pigment dispersion within the melanophore, it is evident that synthesis of melanin is occurring independently of the state of pigment expansion or contraction, whether naturally occurring or artificially induced.

Correlation of sojourn on black or white background with the incorporation of tyrosine into melanin indicated that a four-hour incubation period on either background, under conditions of constant illumination, yielded melanin with similar levels of radioactivity. Furthermore, this level was not different from that found in crabs maintained for 24 hours under conditions of natural illumination. Crabs maintained in total darkness showed a level of incorporation higher than the corresponding animals under normal lighting conditions. However, this result should be re-investigated because of the small number of crabs forming this sample.

Inhibition of tyrosine incorporation

Phenylthiourea has been used successfully as an *in vivo* inhibitor of melanin formation in rats (Dieke, 1947), in ascidian embryos (Whittaker, 1960), in squid embryos and freshly-hatched squids (Arnold, personal communication; Fitzpatrick *et al.,* 1961). The effect of PTU is reversible since pigmentation resumes after withdrawal of the compound (Fitzpatrick *et al.,* 1958; Arnold, personal communication). In the fiddler crab, PTU has no demonstrable effect on the state of pigment dispersion within the chromatophores; therefore, it must be acting as an inhibitor of the tyrosine-tyrosinase reaction rather than having some secondary effect on the melanophore. The radioactivity of the melanin extracted from destalked crabs which received 0.5 mg. PTU per gram wet weight was reduced to a value one-third of that of the non-inhibited destalked animals. In normal crabs inhibited with PTU a 50% reduction in melanin label occurred. In an experiment comparing normal and destalked crabs and their responses to PTU, the level of radioactivity of the melanin extracted from destalked crabs treated with PTU was the same as the level of radioacivity of the melanin extracted from normal crabs treated with PTU. Further analysis would be necessary to decide whether or not this correspondence between levels of radioactivity of pigment in destalked and normal crabs treated with PTU is coincidental.

Factors relating to pigment destruction

Animals injected with 0.5 μC. of tyrosine-C^{14} and maintained for 24 hours on a neutral background with natural lighting were utilized as controls in the following experiments. Animals similarly treated were then transferred to white or black backgrounds, first for 72 hours and in a subsequent experiment for up to 192 hours. The first experiment indicated that 96% of the label was lost from the melanin of pre-labeled crabs maintained on a white background for 72 hours. The subsequent experiments revealed that the loss of the label from melanin appeared to occur in an exponential fashion for the first 96 hours. Fifty per cent of the label was lost 48 hours after transfer to a white background. Animals maintained on a black background showed no loss of label from melanin after 72 hours. Pre-labeled crabs maintained on a white background for four hours exhibited no measurable destruction of pigment.

The term "albedo" is often used in discussions concerning background responses. It has been classically defined as the ratio of reflected to incident light. The albedo has been postulated as a controlling factor in morphological color changes that occur in fish and in amphibians (Sumner, 1940). For example, animals maintained on a white background will be under conditions of high albedo, whereas those on a black background will be under conditions of low albedo. In the present work, animals on a black background illuminated from below will experience a high albedo. Consequently this treatment is comparable to a white background illuminated from above, from this point of view. Crabs under the above situation compensate for the illumination from below and continue to behave as if they were on a black background illuminated from above. This indicates that an albedo effect (in this simple sense) on pigmentation is probably not the main controlling factor in morphological color changes in the fiddler crab.

In this paper I have tried to elucidate some features of morphological color changes in Crustacea. Early workers in this field worked mainly with fish and amphibians (see review by Sumner, 1940). These workers all seem to accept Babak's hypothesis that factors which promote expansion of the pigment within the chromatophore also promote synthesis of new pigment and formation of new chromatophores. Continued contraction of the pigment within the chromatophores, on the other hand, produces the reverse effect. Those workers making observations on morphological color changes in Crustacea also seem to accept the above hypothesis.

The present work shows that the incorporation of added tyrosine into the black pigment, melanin, occurs independently of the state of pigment expansion or contraction within the chromatophores and, further, that it occurs independently of background shade. The newly formed pigment may be stable over long periods of time.

This discussion assumes that all melanin synthesis occurs within melanophores capable of physiological color changes. If melanin synthesis occurred in other epidermal cells, Babak's hypothesis would not be applicable. Green (1964) has shown that in a related crab, *Ocypode ceratophthalma*, the pigment of newly appearing black chromatophores is capable of dispersal and contraction. However, localization of the precise site of melanin synthesis in the fiddler crab, *Uca pugnax*, will require additional investigation.

If we re-evaluate Babak's hypothesis in terms of the present observations, we must still admit the possibility of a correlation between the state of pigment dispersion and the rate of pigment synthesis. However, if this correlation is to be maintained, the control of synthesis rate must reside in the control of the level of available tyrosine which can enter the synthesis pathway rather than any direct control over the pathway itself. No information is available concerning the level of free tyrosine in the fiddler crab.

On the other hand, destruction of melanin can be induced by placing the animals on a white background. This occurs despite the fact that the changes induced by this procedure in the state of pigment dispersion are not marked (Brown and Sandeen, 1948; Brown and Hines, 1952). Consequently, the evidence which is available does not support a detailed correspondence between physiological and morphological color changes which would justify invoking the same control system for both phenomena.

I would like to acknowledge the helpful suggestions and pertinent criticisms of my advisor, Professor Grover C. Stephens. Dr. George Szabó of the Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts, generously gave me sufficient dihydroxyphenylalanine-C^{14} to carry out the reported experiments.

SUMMARY

1. The *Uca* black chromatophore pigment has been identified as a melanin on the basis of: (1) solubility and bleaching reactions; (2) incorporation of tyrosine and dihydroxyphenylalanine into the pigment; and (3) inhibition of tyrosine incorporation by phenylthiourea, a known inhibitor of melanin synthesis.

2. The incorporation of tyrosine-C^{14} into *Uca* melanin is linearly related to micrograms tyrosine injected in the range 0.88–208.00 μg. tyrosine per gram wet weight.

3. Melanin synthesis occurs rapidly under normal conditions and may be related to the influx of free tyrosine.

4. The incorporation of tyrosine-C^{14} into *Uca* melanin is temperature-dependent; the energy of activation, as calculated from the Arrhenius plot of the data, is 5757 calories.

5. Melanin, once labeled, retains the label for considerable periods of time, thereby indicating the stability of the pigment under these conditions.

6. Destalked crabs under similar conditions have a level of tyrosine incorporation approximately 70% higher than do normal animals. This may reflect the increased activity of the epidermis following eye-stalk ablation.

7. Melanin synthesis occurs independently of the state of pigment dispersion, whether naturally-occurring or artificially-induced.

8. Normal animals maintained on a white background under constant illumination show exponential destruction of melanin with a half-time of 48 hours.

9. The available evidence does not support a detailed correspondence between physiological and morphological color changes which would justify invoking the same control systems for both phenomena.

LITERATURE CITED

ABRAMOWITZ, A. A., 1937. The chromatophorotropic hormone of the Crustacea. *Biol. Bull.*, **72**: 344–365.

ATTIE, J. N., AND R. A. KHAFIF, 1964. Melanotic Tumors: Biology, Pathology, and Clinical Features. C. C Thomas, Springfield. Chapter I.

BABAK, E., 1912. Über den Einfluss des Lichtes auf die Vermehrung des Hautchromatophoren. *Arch. ges. Physiol.*, **149**: 462–470.

Bliss, D. E., 1953. Endocrine control of metabolism in the land crab, *Gecarcinus lateralis* (Fréminville). I. Differences in the respiratory metabolism of sinusglandless and eyestalkless crabs. *Biol. Bull.*, **104**: 275–296.

Bowman, T. E., 1942. Morphological color change in the crayfish. *Amer. Nat.*, **76**: 332–336.

Brown, F. A., Jr., 1934. The chemical nature of the pigments and the transformations responsible for color changes in *Palaemonetes*. *Biol. Bull.*, **67**: 365–380.

Brown, F. A., Jr., 1940. The crustacean sinus-gland and chromatophore activation. *Physiol. Zoöl.*, **13**: 343–355.

Brown, F. A., Jr., and M. N. Hines, 1952. Modification in the diurnal pigmentary rhythm of *Uca* effected by continuous illumination. *Physiol. Zoöl.*, **25**: 56–70.

Brown, F. A., Jr., and M. I. Sandeen, 1948. Responses of the chromatophores of the fiddler crab, *Uca*, to light and temperature. *Physiol. Zoöl.*, **21**: 361–371.

Brown, F. A., Jr., and H. M. Webb, 1948. Temperature relations of an endogenous daily rhythmicity in the fiddler crab, *Uca*. *Physiol. Zoöl.*, **21**: 371–381.

Carlisle, D. B., and F. Knowles, 1959. Endocrine Control in Crustacea. Cambridge University Press, Cambridge, England.

Carlson, S. P., 1935. The color changes in *Uca pugilator*. *Proc. Nat. Acad. Sci.*, **21**: 549–551.

Dieke, S. H., 1947. Pigmentation and hair growth in black rats, as modified by the chronic administration of thiourea. *Endocrinology*, **40**: 123.

Edwards, G. A., 1952. The influence of eyestalk removal on the metabolism of the fiddler crab. *Physiol. Comp. Occol.*, **2**: 34–50.

Fingerman, M., C. Oguro and M. Miyawaki, 1964. Relationship between melanophore response in the fiddler crab, *Uca pugnax*, and dosage of sinus gland and central nervous organ extracts. *Physiol. Zoöl.*, **37**: 83–89.

Fitzpatrick, T. B., P. Brunet and A. Kukita, 1958. The nature of hair pigment. *In:* The Biology of Hair Growth. W. Montagna and R. A. Ellis, Editors. Academic Press, New York. Chapter 13.

Fitzpatrick, T. B., M. Seiji, R. Simpson and G. Szabó, 1961. Studies of melanin biosynthesis in the ink sac of the squid (*Loligo pealii*). *Biol. Bull.*, **121**: 389–390.

Forrest, H. S., 1959. The ommochromes. *In:* Conference on Biology of Normal and Atypical Pigment Cell Growth. M. Gordon, Editor. Academic Press, N. Y., pp. 619–630.

Gortner, R. A., 1910. Studies on melanin. I. Method of isolation; the effect of alkali on melanin. *J. Biol. Chem.*, **8**: 341–363.

Gould, B. S., 1939. The nature of animal and plant tyrosinases. *Enzymologia*, **7**: 292–296.

Green, J. P., 1963. An analysis of morphological color change in two species of brachyuran crustaceans. Ph.D. Thesis, University of Minnesota, Minneapolis.

Green, J. P., 1964. Morphological color change in the Hawaiian ghost crab, *Ocypode ceratophthalma* (Pallas). *Biol. Bull.*, **127**: 407–413.

Guyselman, J. B., 1953. An analysis of the molting process in the fiddler crab, *Uca pugnax*. *Biol. Bull.*, **104**: 115–137.

Hogben, L. T., and D. Slome, 1931. The pigmentary effector system. VI. The dual character of endocrine coordination in amphibian colour change. *Proc. Roy. Soc. London, Ser. B*, **108**: 10–53.

Kincaid, F. D., and B. T. Scheer, 1952. Hormonal control of metabolism in crustaceans. IV. Relation of tissue composition of *Hemigrapsus nudus* to intermolt cycle and sinus gland. *Physiol. Zoöl.*, **25**: 371–380.

Lea, A. J., 1945. A neutral solvent for melanin. *Nature*, **156**: 478.

Lerner, A. B., and J. D. Case, 1959. Pigment cell regulatory factors. *J. Invest. Dermatol.*, **32**: 211–221.

Lerner, A. B., and T. B. Fitzpatrick, 1950. Biochemistry of melanin formation. *Physiol. Rev.*, **30**: 91–126.

Lerner, A. B., T. B. Fitzpatrick, E. Calkins and W. H. Summerson, 1949. Mammalian tyrosinase: Preparation and properties. *J. Biol. Chem.*, **178**: 185–195.

Lison, L., 1936. Histochemie Animale. Gauthier-Villars, Paris.

Mason, H. S., 1948. The chemistry of melanin. III. Mechanism of the oxidation of DOPA by tyrosinase. *J. Biol. Chem.*, **172**: 83–99.

Megusar, F., 1912. Experimente über den Farbwechsel der Crustaceen. (I. *Gelasimus*. II. *Potamobius*. III. *Palaemonetes*. IV. *Palaemon*.). *Arch. f. Entw.*, **33**: 462–665.

NEEDHAM, A. E., AND P. C. J. BRUNET, 1957. The integumental pigment of *Asellus. Experientia,* **13**: 207–209.

NEILAND, K. A., AND B. T. SCHEER, 1953. The influence of fasting and of sinus gland removal on body composition of *Hemigrapsus nudus.* (Part V of: The hormonal regulation of metabolism in crustaceans.). *Physiol. Comp. Oecol.,* **3**: 321–326.

PEARSE, A. G. E., 1961. Histochemistry. Little, Brown and Company, Boston.

SANDEEN, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator,* with special reference to their origins and actions. *Physiol. Zoöl.,* **23**: 337–352.

SCHWABE, C. W., B. T. SCHEER AND M. A. SCHEER, 1952. The molt cycle in *Panulirus japonicus.* (Part II of: The hormonal regulation of metabolism in crustaceans.). *Physiol. Comp. Oecol.,* **2**: 310–320.

SEIJI, M., K. SHIMAO, M. S. C. BIRBECK AND T. B. FITZPATRICK, 1963. Subcellular localization of melanin biosynthesis. *Ann. N. Y. Acad. Sci.,* **100**: 497–533.

STEPHENS, G. C., 1957. Twenty-four hour cycles in marine organisms. *Amer. Nat.,* **91**: 135–152.

SUMNER, F. B., 1940. Quantitative changes in pigmentation resulting from visual stimuli in fishes and amphibia. *Biol. Rev.,* **15**: 351–375.

WHITTAKER, J. R., 1960. An *in vitro* analysis of tyrosinase function and melanin formation in ascidian embryos. *Anat. Rec.,* **138**: 388–389.

ANAEROBIC GLYCOLYSIS IN AMPHIBIAN DEVELOPMENT.[1] HOMOGENATES

JOHN R. GREGG, JANE J. MacISAAC AND MARY ANN PARKER

Department of Zoology, Duke University, Durham, North Carolina

Hybrid frog embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm do not develop normally beyond the stage at which the dorsal lip of the blastopore is first established (Moore, 1946). Morphologically, the failure to continue gastrulation has been traced to the inability of the chordamesoderm to undergo convergent extension and to its abnormal behavior in relation to tissues of other germ layers (Gregg and Klein, 1955). The only metabolic peculiarity known to antedate gross morphological failure is the deficient rate, relative to that of normal *R. pipiens* controls, at which lactic acid is produced under anaerobiosis (L. G. Barth, 1946; Gregg, 1962). In the sequel, we shall attempt to establish that this is not due to a paucity of the enzymatic components of glycolysis, and to present a view of the energetics of hybrid development which is as consistent as possible with the information presently available.

METHODS

Embryological

Developing embryos were obtained by stripping eggs from pituitary-activated *R. pipiens* females into suspensions of active *R. pipiens* or *R. sylvatica* sperm. After an hour or so, the clutches were cut into small groups of 20–40 embryos and distributed into several large fingerbowls, each containing 100–200 ml. of 10% amphibian Ringer's solution, without phosphate or bicarbonate. Development was allowed to proceed, usually in an incubator maintained at 10° C., but sometimes at room temperature. The medium was renewed at intervals. Under these conditions, frog embryos develop aerobically, unattended by the formation of any but negligible quantities of lactic acid (Gregg, 1962). Before use, the embryos were freed of their jelly coats with the aid of jeweller's forceps.

Chemical

For the preparation of cell-free homogenates, the embryos were washed several times with cold saline-buffer solution (see below), and subjected to the action of a power-driven homogenizer. Incorporation of glycolytic cofactors and substrates yielded a system of the following composition, similar to that developed by A. I. Cohen (1954):

---

[1] This work has been supported in part by a research gront, No. A 2146, from the Public Health Service. The authors have benefited from the assistance of Harry T. Klugel III.

Saline-buffer (pH 7.5)

Potassium bicarbonate: 2×10^{-2} *M*
Magnesium chloride: 4×10^{-3} *M*
Potassium chloride: 1.5×10^{-2} *M*
Potassium phosphates: 2×10^{-2} *M*

Cofactors

AMP: 10^{-3} *M*
ATP (dipotassium): 10^{-3} *M*
DPN: 5×10^{-4} *M*

Substrate (one only)

Glycogen: 1%
Others (see RESULTS): 2×10^{-3} *M*

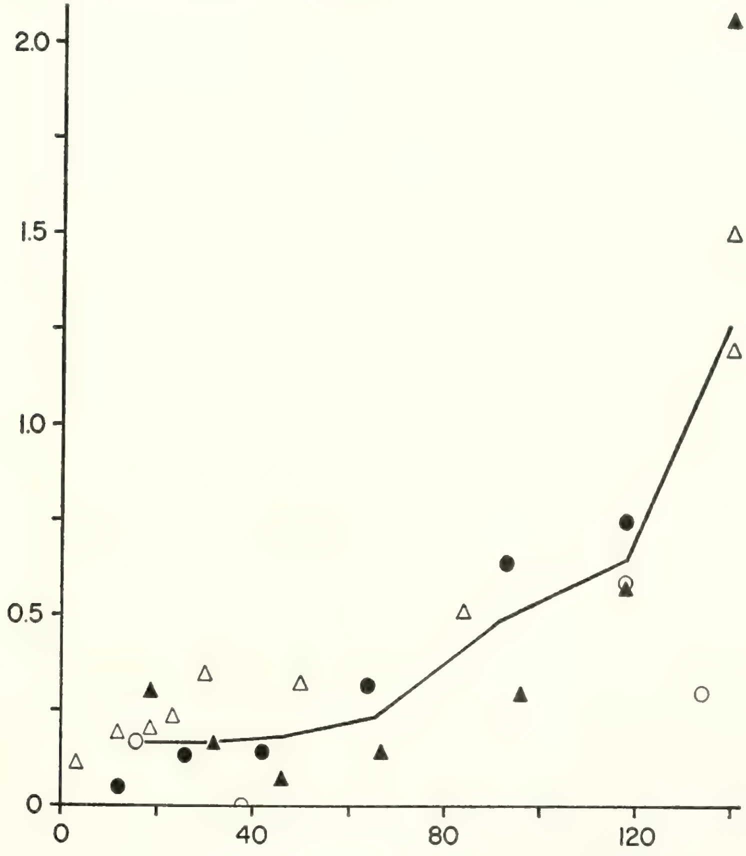


FIGURE 1. Lactic acid in fresh homogenates, *R. pipiens.* ●, Clutch 11.27.2; ▲, Clutch 1.28.3; ○, Clutch 2.7.3; △, Clutch 2.18.3. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo, 24° C. The solid line connects averages for the age-intervals 0–20, 21–40, . . ., 121–140 hours.

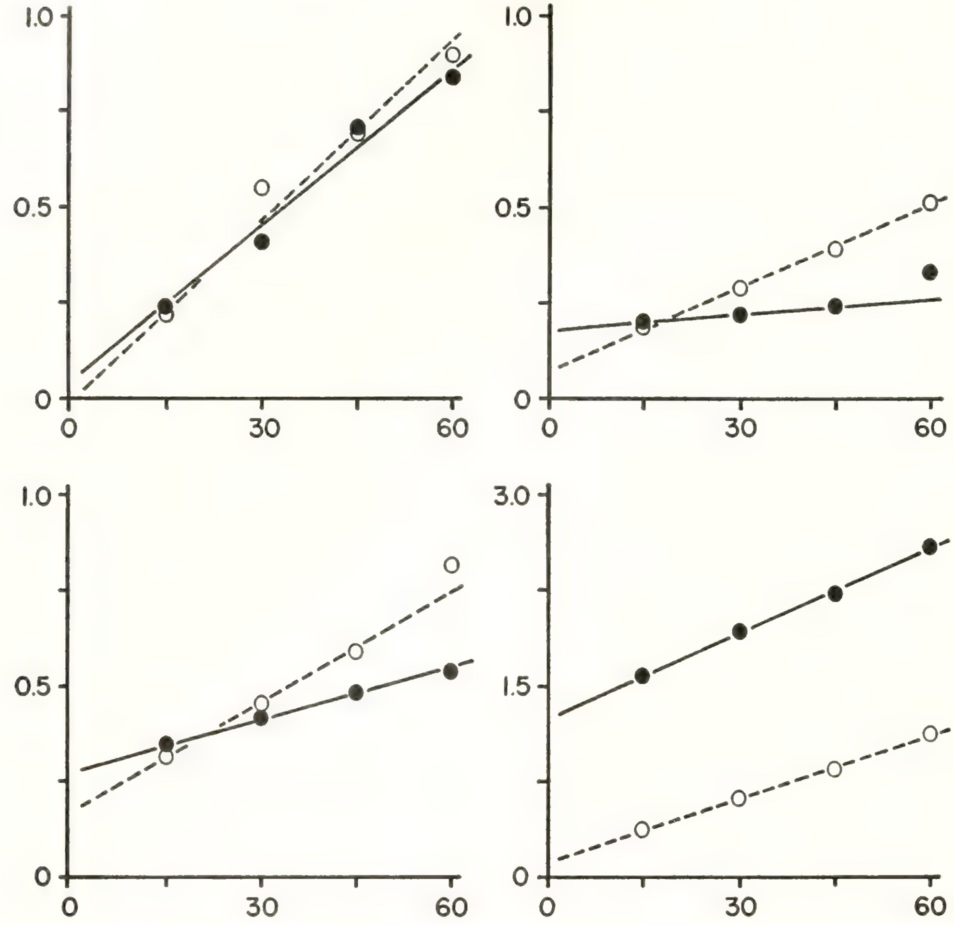


FIGURE 2. Time course of lactic acid production by anaerobic homogenates. Clutch 4.19.3. Solid lines, *R. pipiens*. Broken lines, hybrids. Substrate, glycogen. Upper left, standard age 4 hours. Upper right, standard age 56 hours. Lower left, standard age 84 hours. Lower right, standard age 140 hours. Abscissae, minutes. Ordinates, μg. lactic acid per embryo, 24° C.

Homogenized embryos: 10 per ml.

Yeast hexokinase was added in some experiments (see RESULTS, *Glucose*) to a final concentration of 0.5 mg. (27 KM units) per ml. The enzyme and all cofactors and substrates, excepting glucose, were obtained from Mann Research Laboratories, Inc., New York City, and were made up in fresh solutions for each experiment.

Anaerobiosis was obtained with the help of the apparatus previously described (Gregg, 1962), consisting of a train of flasks, each containing 2 ml. of the homogenate system, continuously flushed with 95% N_2: 5% CO_2 and maintained in a shaking bath at 24° C. At the ends of suitably chosen intervals, flasks were removed from the distal end of the train, 0.5 ml. of 30% trichloroacetic acid added to each, and protein-free extracts obtained by centrifuging. The amounts of lactic acid present in the extracts were determined by a modification (Gregg, 1962) of the method of Barker and Summerson (1941). (In the former publication, the color reagent employed, *p*-phenylphenol, was incorrectly specified as polyindophenol; and the colorimeter used, a Bausch and Lomb Spectronic 20, was mistakenly credited to Beckman.)

Terminological

Developmental stages were determined by reference to the drawings of Shumway (1940), which standardize the course of *R. pipiens* development at 18° C. Regardless of their actual thermal histories, embryos in a given Shumway stage are assigned the corresponding standard age in hours. Hybrid embryos are assigned the standard ages of *R. pipiens* control embryos of the same thermal history and female parentage, and are said to be in stage H-*s* when their controls are in stage *s*.

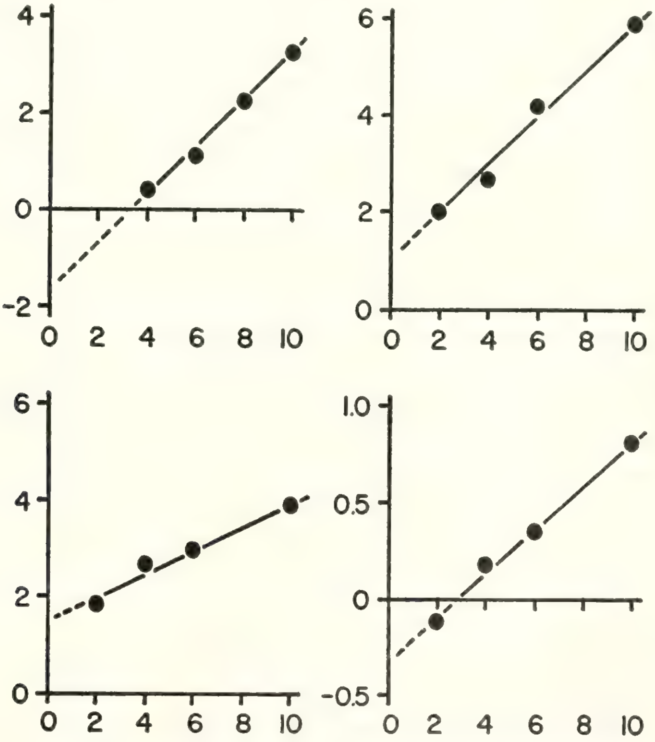


FIGURE 3. Rates of lactic acid production by anaerobic homogenates (*R. pipiens*) of different concentrations. Upper left, clutch 4.23.4; standard age, one hour; substrate, glycogen. Upper right, clutch 3.24.3; standard age, 38 hours; substrate, fructose-1,6-diphosphate. Lower left, clutch 3.24.3; standard age, 67 hours; substrate, fructose-1,6-diphosphate. Lower right, clutch 2.7.3; standard age, 151 hours; substrate, glycogen. Abscissae, embryos per ml. Ordinates, μg. lactic acid per hour, 24° C.

RESULTS

General characteristics of the system

We have already mentioned that intact aerobic embryos contain lactic acid in such small amounts as to be safely ignored. This convenience is not forthcoming in work with homogenates. Figure 1 shows that the amount of lactic acid present in freshly made homogenates is an increasing function of the age of the embryos from which the homogenates are made (*R. pipiens*). These amounts of lactate are not present in intact embryos; therefore, they must be formed very rapidly (within a few minutes) after homogenization. It is unlikely that they are formed from the exogenous substrates added to the homogenates, nor do these substrates themselves yield any detectable color in the lactic acid determinations, but the actual source is unknown. Extrapolation of the graphs of Figure 2 suggests that the factor of initial lactic acid is of much less importance in homogenates of hybrid embryos, but we have made no direct determinations.

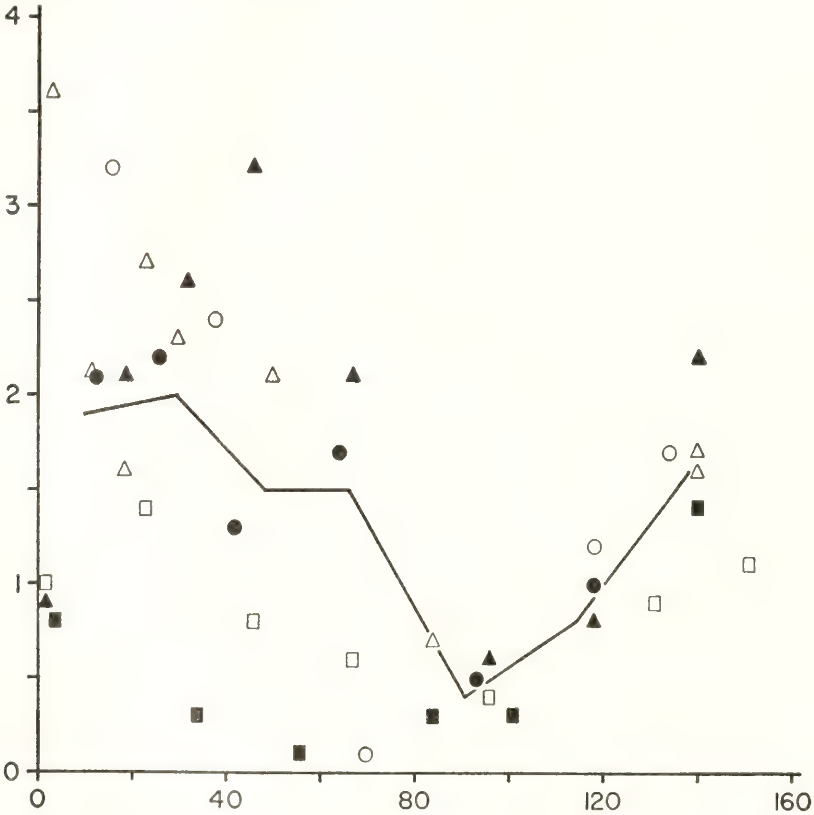


FIGURE 4. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. ●, clutch 11.27.2; ▲, clutch 1.28.3; ○, clutch 2.7.3; △, clutch 2.18.3; ■, clutch 4.19.3; □, clutch 5.22.3. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, µg. lactic acid per embryo per hour, 24° C. The solid line connects averages for the age-intervals 0–20, 21–40, . . ., 121–140 hours.

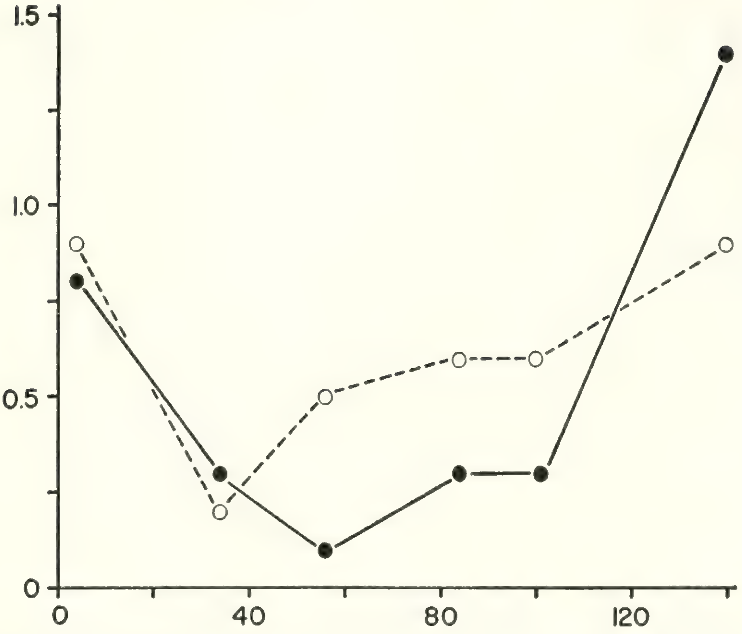


FIGURE 5. Rates of lactic acid production by anaerobic homogenates. Clutch 4.19.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

In special experiments, we have found that a linear production of lactic acid commences within three minutes after the onset of gassing and persists for at least two hours. Our routine procedure, however, is implicit in the graphs of Figure 2, which also show how the factor of initial lactic acid was controlled. For each experiment, enough homogenate was prepared to load four flasks in the gassing train. At intervals, flasks were removed from the train and the amounts of lactic acid present were determined. A graph relating the amount of lactic acid present to the time spent under anaerobiosis was then prepared, and the slope of the line obtained was used to calculate the rate of glycolysis. Departures from the principles of this procedure will be mentioned explicitly in the appropriate contexts.

All else being equal, generalization of the results obtained with the homogenate technique is most feasible when the activity being measured is a linear function of the amount of homogenized tissue present. Attempts to establish that this desideratum is satisfied by our own homogenate systems have not yielded altogether satisfactory results (Fig. 3); for, although the graphs relating the number of homogenized embryos per ml. of homogenate to the glycolytic rate exhibited are linear, as we hoped, they do not pass through the origins. We have not been able to overcome this difficulty, and the reasons for it are not clear. Our other data, therefore, must be regarded as holding only for the standard homogenate concentration (10 embryos per ml.) that we have employed.

Finally, we should mention that the glycolytic rates exhibited by homogenates from which exogenous substrates have been omitted are very low. This is shown

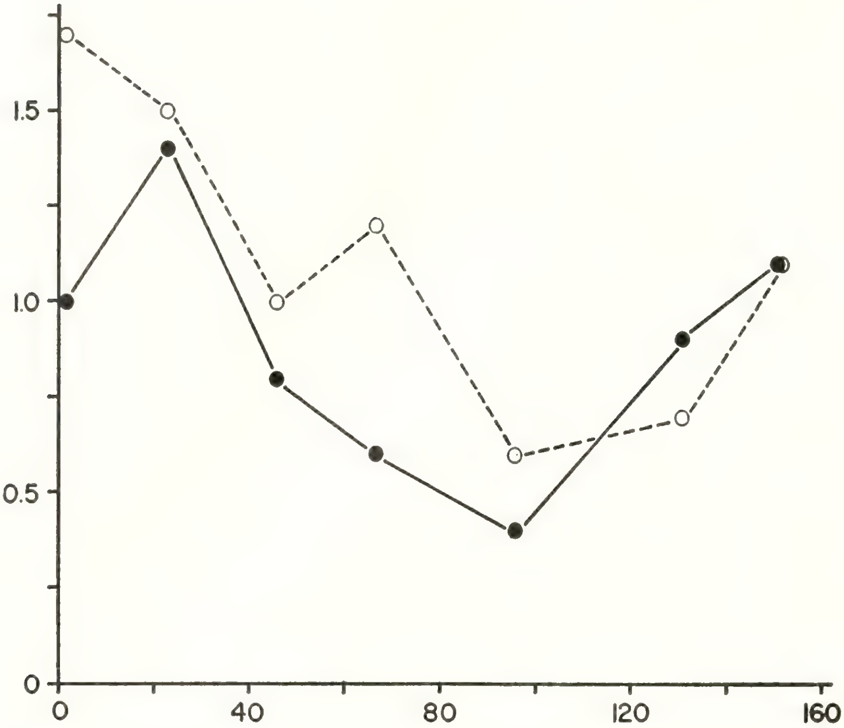


FIGURE 6. Rates of lactic acid production by anaerobic homogenates. Clutch 5.22.3. Solid line, *R. pipiens,* broken line, hybrids. Substrate, glycogen. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

explicitly by Figure 9, and is implicit in the data of Figure 8 and Figure 11. Similar results were reported by A. I. Cohen (1954).

Glycogen

A total of six clutches of homogenized *R. pipiens* embryos have been systematically assayed to establish the relation between age and glycolytic activity in the presence of exogenous glycogen. The results are collected in Figure 4. The developmental patterns of glycolysis exhibited are variable, especially those of homogenates from embryos younger than 80 to 90 hours (standard age). Perhaps the best generalization that the data will support is that the glycolytic rate falls off during the transition from fertilized egg to tailbud embryo, and then rises again during the remainder of pre-hatching development. The variability exhibited probably is not an artifact of the experimental procedures to which the homogenates were subjected, but more likely is due to uncontrollable non-uniformity in the preparation of homogenates or to intrinsic variability in the clutches themselves.

Embryos of two of these clutches were controls for hybrid embryos, with which they are compared in Figures 5 and 6. Clearly, the subnormal glycolytic rates of intact hybrids (L. G. Barth, 1946; Gregg, 1962) are not characteristic of their homogenates. It appears, therefore, that they have a normal complement of the enzymes required for the transformation of glycogen into lactic acid; and it will be seen that this conclusion is supported by the remainder of the relevant data in this paper.

Glucose-1-phosphate (dipotassium)

Results similar to those obtained with glycogen are observed when the homogenates are supplied with exogenous glucose-1-phosphate (Fig. 7). We prefer not to emphasize the apparent superior ability of the hybrid homogenates to glycolyze this substrate. The only point we wish to make is that the data indicate that hybrids are not less well supplied than their normal controls with the enzymes required to transform glucose-1-phosphate into lactic acid.

Glucose-6-phosphate (disodium)

Figure 8 exhibits the relation between age and glycolytic activity in the presence and absence of exogenous glucose-6-phosphate. The factor of initial lactic acid was not controlled in these experiments, and this probably accounts for the apparent rise in the rate of endogenous glycolysis in the normal controls. Taking this into account, however, it is clear that homogenates unfortified with exogenous substrates exhibit very low rates of glycolysis. The rate at which exogenous glucose-6-phosphate is utilized for the production of lactic acid appears to increase steadily throughout development (the low value for *R. pipiens* at 112 hours was obtained with embryos that were visibly less well homogenized than usual), unlike the rates

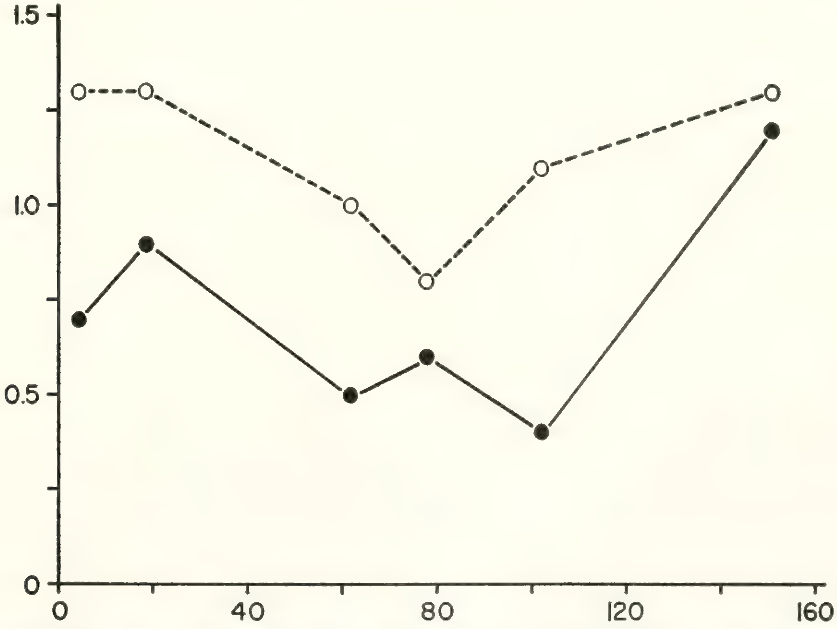


FIGURE 7. Rates of lactic acid production by anaerobic homogenates. Clutch 4.24.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, glucose-1-phosphate. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

obtained with glycogen and with other phosphorylated hexoses of the Embden-Meyerhof system. What this means is not clear; we shall remark only that hybrids do not seem to be significantly less well endowed than their normal controls with the enzymes required to transform glucose-6-phosphate into lactic acid.

Fructose-1,6-diphosphate (magnesium)

The rates at which homogenized *R. pipiens* embryos of various ages produce lactic acid in the presence and absence of exogenous hexose diphosphate are exemplified in Figure 9. As usual, the endogenous rates are very low, and glycolysis is stimulated by the presence of the exogenous substrate. Figure 10 shows that homogenized hybrids do not differ significantly from homogenized *R. pipiens* controls in the rates at which they glycolyze hexose diphosphate; as before, this is interpreted to mean that the relevant enzymes are present in normal amounts.

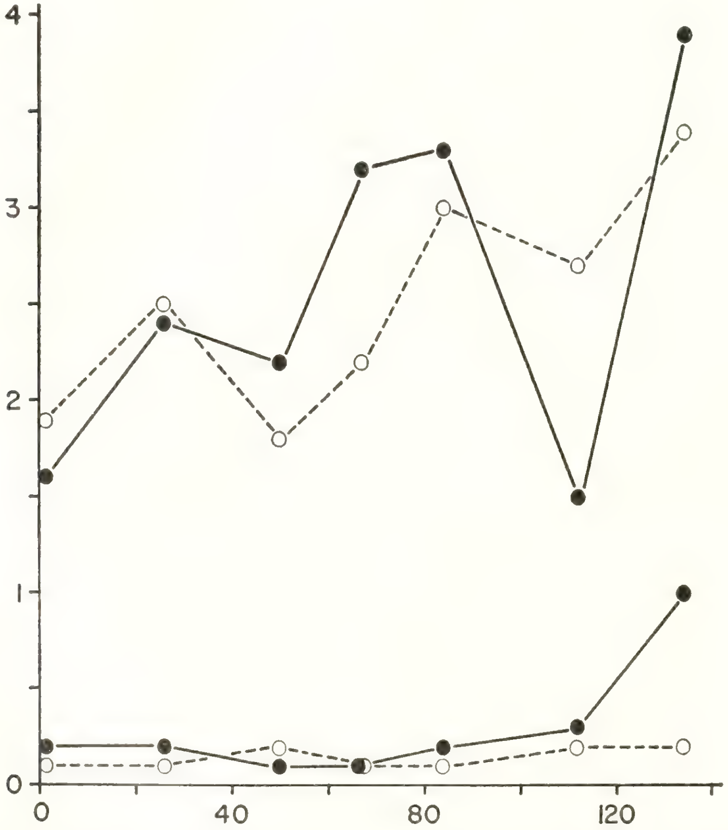


FIGURE 8. Rates of lactic acid production by anaerobic homogenates. Clutch 5.12.4. Solid lines, *R. pipiens*. Broken lines, hybrids. Substrate, upper two curves, glucose-6-phosphate. Substrate, lower two curves, endogenous. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C. In these experiments, the factor of initial lactic acid has not been controlled (see *General characteristics of the system*).

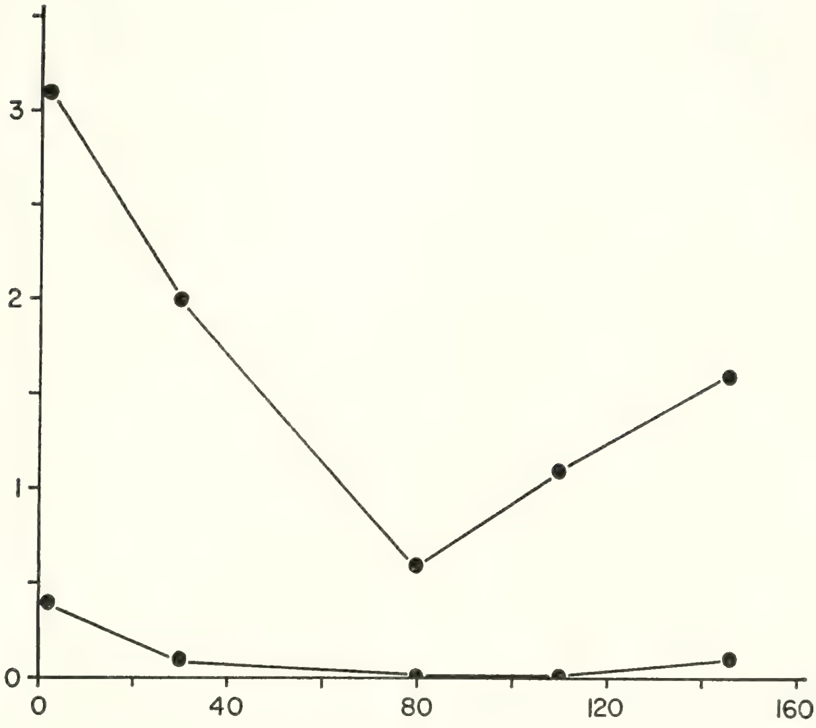


FIGURE 9. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. Clutch 4.8.3. Substrate, upper curve, fructose-1,6-diphosphate. Substrate, lower curve, endogenous. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

Glucose

Free reducing sugars are not present in *R. pipiens* embryos (Gregg, 1948), and are unavailable, therefore, as normal endogenous sources of energy. Furthermore, A. I. Cohen (1954) has reported that *R. pipiens* homogenates are unable to transform exogenous glucose into lactic acid, and this result is confirmed by our own data (Fig. 11). Cohen suggested that the inactivity of glucose might be due to the absence of hexokinase, but the situation appears to be somewhat more complex; for, as Figure 11 shows, addition of yeast hexokinase along with the exogenous glucose results in but negligible stimulation of lactic acid production, while the addition of hexokinase alone is a powerful stimulant to glycolysis. If the enzyme is inactivated by heat prior to adding it to the homogenates, it is without effect. At the moment, we cannot explain this remarkable state of affairs. It is not an artifact of the clutch of embryos represented in Figure 11, because we have observed it in all of the clutches that we have examined. It is unlikely that glucose and some endogenous substrate competitively inhibit one another, because inhibition would have to be mutually complete, and this does not conform to the patterns known for yeast hexokinase (Slein *et al.*, 1950). Further investigation is scheduled for next season.

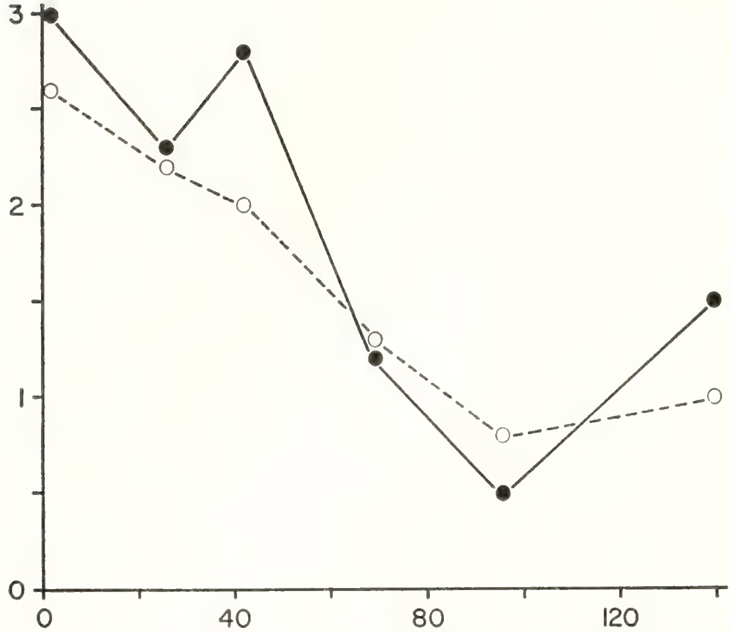


FIGURE 10. Rates of lactic acid production by anaerobic homogenates. Clutch 4.18.3. Solid line, *R. pipiens*. Broken line, hybrids. Substrate, fructose-1,6-diphosphate. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C.

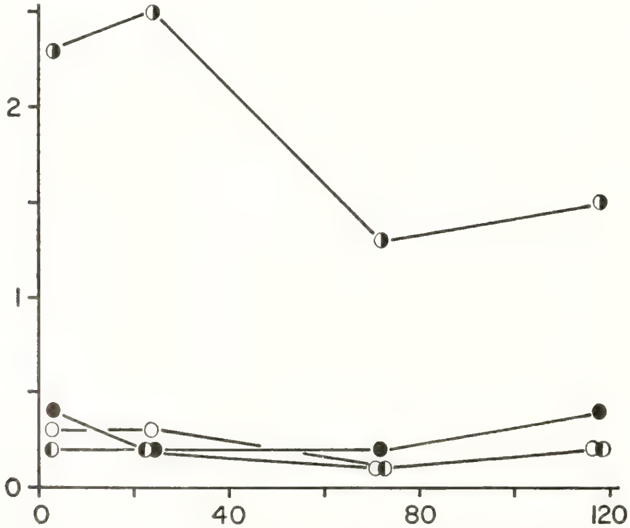


FIGURE 11. Rates of lactic acid production by anaerobic homogenates, *R. pipiens*. Clutch 4.17.4. ●, substrate glucose plus yeast hexokinase; ◐, substrate glucose, no yeast hexokinase; ◑, substrate endogenous plus yeast hexokinase; ○, substrate endogenous, no yeast hexokinase. Abscissa, standard age in hours, 18° C. Ordinate, μg. lactic acid per embryo per hour, 24° C. In these experiments, the factor of initial lactic acid has not been controlled (see *General characteristics of the system*).

Discussion

An abstract model of the energetics of developmental processes appears in Figure 12. On the right is represented an arbitrary endergonic developmental state-transformation; on the left, an arbitrary exergonic process in which some substrate is oxidized, in the presence of oxygen, to carbon dioxide and water, or, anaerobically, to lactic acid. The two processes are shown coupled by and to a typical energy transfer and storage system. The model implies that activity of the entire system might be lowered by internal defects in any of the three types of processes represented, or by defective coupling between them.

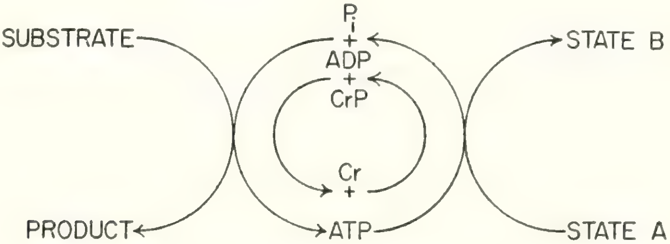


Figure 12. Abstract model of the energetics of developmental processes. Cr, creatine; CrP, creatine phosphate.

Up to stage H-10, when gross morphological failure occurs, hybrid embryos respire at a normal rate (L. G. Barth, 1946), have a normal respiratory quotient (L. G. Barth, 1946), utilize stored glycogen slightly faster than normal embryos (Gregg, 1948), but are already deficient in the rate at which they produce lactic acid anaerobically (L. G. Barth, 1946; Gregg, 1962). Beyond stage H-10, they respire at increasingly subnormal rates (L. G. Barth, 1946; Gregg, 1960), exhibit, beginning at stage H-11 or H-12, abnormally high respiratory quotients (L. G. Barth, 1946), utilize subnormal amounts of stored glycogen (Gregg, 1948), and are increasingly subnormal in the capacity to produce lactic acid anaerobically (L. G. Barth, 1946; Gregg, 1962).

There is some evidence to indicate that the respiratory deficiency mentioned may be internal to the system of substrate-product transformations. Malonic acid in fairly strong concentration (0.04 *M*) is known to inhibit gastrulation and to reduce the respiration of gastrula explants (Ornstein and Gregg, 1952), presumably by inhibiting the conversion of succinic acid to fumaric acid. S. Cohen (1963) has discovered that hybrids accumulate abnormal amounts of malonic acid during the establishment of the block to morphogenesis at stage H-10. If it could be shown that there is sufficient malonic acid to inhibit the production of energy by respiratory processes, then much of what is known of the energetics of hybrid development would be understandable. We should expect, however, to find an accumulation of succinic acid accompanied by an abnormally low content of fumaric acid; but as far as one can tell from Cohen's chromatographs, both of these metabolites are present in relative normal concentrations. Furthermore, we should expect to find no increase in respiratory rate attendant upon chemical uncoupling of respiration and phosphorylation; but it has been shown that in the presence of the uncoupling agent 2,4-dinitrophenol the respiration of blocked hybrids is tripled or quadrupled (Gregg, 1960). This suggests that the hybrid respiratory system is not held at a subnormal level of activity by accumulated inhibitory metabolites, and that it is capable of delivering much more energy than is demanded of it ordinarily. But in their turn, the results with 2,4-dinitrophenol suggest some internal restraints in the respiratory systems of post stage H-10 hybrids, for the respiratory rates under maximal stimulation by the uncoupling agent become progessively subnormal, relative to those of *R. pipiens* controls under similar treatment. The restraints are probably organizational in nature, unaccompanied by quantitative deficiencies of respiratory components, because homogenates of hybrid embryos at all stages respire at the same rates as homogenates of *R. pipiens* controls (Gregg and Ray, 1957). To this finding, we may now add the results of the present work, which indicate in a similar way that the subnormal capacity of intact hybrids to utilize glycogen and to produce lactic acid anaerobically is not the result of a relative deficiency of glycolytic enzymes.

Prior to stage H-10, hybrid embryos exhibit a normal aerobic phosphate balance in respect to total phosphorus, total acid-soluble phosphorus, ATP, creatine phosphate and inorganic phosphorus (Barth and Jaeger, 1947; Gregg and Kahlbrock, 1957; Harrison, 1963). Beyond stage H-10, however, they exhibit somewhat less inorganic phosphorus than normal controls, which may indicate that they meet the energy-demands of their curtailed transformations of state a little better than normal embryos meet theirs. Some limitation upon their ability to store phosphate bond energy appears after stage H-13, because the rate at which creatine is synthesized falls away from the normal, and this is attended by increasingly subnormal stores of creatine phosphate (Harrison, 1963).

During relatively long periods of anaerobiosis (10–22 hours) the level of inorganic phosphorus is elevated beyond that which can be accounted for by the observed depletion of ATP, both in hybrids and in their normal controls (gastrulae). Barth and Jaeger (1947), who made this discovery, suggested that the excess inorganic phosphorus appearing might have its source in creatine phosphate. For short periods of anaerobiosis, Harrison (1963) has shown, indeed, that the inorganic phosphorus appearing is exactly accounted for by creatine phosphate disappearing (hybrids and their controls, all stages); and her results suggest that there is a net breakdown of ATP only when anaerobiosis is further prolonged (*R. pipiens* only, she did not study hybrids under prolonged anaerobiosis).

Analysis of the data of Barth and Jaeger (1947) shows (Gregg, 1957) that there are no statistical differences between the phosphate imbalances exhibited by anaerobic hybrids and their controls (early gastrulae), and this is confirmed by the results of Harrison (1963). With age, however, hybrids and controls exhibit increasingly discrepant responses to two-hour periods of anaerobiosis, control embryos liberating progressively more inorganic phosphorus than hybrids at the expense of creatine phosphate stores (Harrison, 1963). All else being equal, this result is to be expected; for the passage of a normal embryo from an already complex state at age t to an even more complex state at age $t + 1$ should require a greater expenditure of energy than the passage of a blocked hybrid embryo from a less complex state at t to an only slightly more complex state at $t + 1$.

The foregoing discussion, in our opinion, suggests the following general view of the energetics of hybrid development:

(1) There is evidence that organizational restraints internal to the system of substrate-product transformations ultimately limit the rate at which energy can be delivered to the phosphate transfer-storage system.

(2) There is no evidence for defective coupling between the system of substrate-product transformations and the phosphate transfer-storage system.

(3) There is no evidence for internal defects in the phosphate transfer-storage system, other than a somewhat reduced ultimate capacity to store excess energy in creatine phosphate.

(4) There is no evidence for defective coupling between the phosphate transfer-storage system and the system of state-transformations.

(5) There is evidence that the capacity of the system of substrate-product transformations to deliver energy to the phosphate transfer-storage system is not fully exploited; therefore, the primary block to hybrid morphogenesis is not to be ascribed to the ultimate limitations mentioned.

(6) As a working hypothesis, it is suggested that there is a blockage of the system of state-transformations, independent of energy production, transfer or storage; this results, by virtue of the couplings involved, in the lowered rates of energy metabolism that are actually observed. The block may be internal to the system of state-transformations, or may be imposed upon it by the embryonic milieu. In this connection, it is reported that hybrid cells may be made to undergo developmental changes of state that they never exhibit *in situ* by culturing them *in vitro* (L. J. Barth, 1964, p. 71).

SUMMARY

1. Freshly prepared homogenates of *R. pipiens* embryos contain considerable amounts of lactic acid, aerobically produced from an unknown source. The amount present is an increasing function of the age of the embryos from which the homogenates are made.

2. In the presence of suitable exogenous substrates, anaerobic homogenates of *R. pipiens* embryos, and of hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ embryos, produce lactic acid at constant rates for at least two hours. Anaerobic lactic acid production from endogenous substrates occurs at very low rates.

3. The relation between homogenate concentration and the rate of lactic acid production under anaerobiosis is linear, but graphs of the relation do not pass through the origin.

4. Roughly speaking, the rates at which homogenized embryos glycolyze exogenous glycogen, glucose-1-phosphate, glucose-6-phosphate and fructose-1,6-diphosphate are at least as great for hybrid embryos as for normal *R. pipiens* controls.

5. Exogenous glucose is not glycolyzed by homogenized *R. pipiens* embryos, even in the presence of yeast hexokinase. Provided that exogenous glucose is absent, the presence of exogenous hexokinase is a powerful stimulant to the glycolysis of some unidentified endogenous substrate.

6. The energetics of hybrid development is reviewed briefly. It is concluded that the system of state-transformations is blocked in some manner independent of energy production, transfer or storage.

LITERATURE CITED

Barker, S. B., and W. H. Summerson, 1941. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.*, **138**: 535–554.

Barth, L. G., 1946. Studies on the metabolism of development. *J. Exp. Zool.*, **103**: 463–486.

Barth, L. G., and L. Jaeger, 1947. Phosphorylation in the frog's egg. *Physiol. Zool.*, **20**: 133–146.

Barth, L. J., 1964. Development. Selected Topics. Addison-Wesley Publishing Co., Reading, Mass.

Cohen, A. I., 1954. Studies on glycolysis during the early development of *Rana pipiens*. *Physiol. Zoöl.*, **27**: 138–141.

Cohen, S., 1963. $^{11}CO_2$ fixation and the accumulation of malonic acid in amphibian hybrids (*R. pipiens* ♀ × *R. sylvatica* ♂). *Exp. Cell Res.*, **29**: 207–211.

Gregg, John R., 1948. Carbohydrate metabolism of normal and of hybrid amphibian embryos. *J. Exp. Zool.*, **109**: 119–134.

Gregg, John R., 1957. Morphogenesis and metabolism of gastrula-arrested embryos in the hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂. *In:* The Beginnings of Embryonic Development, edited by Albert Tyler, R. C. von Borstel and Charles B. Metz, Publication No. 48 of The American Association for the Advancement of Science. Washington, D. C.

Gregg, John R., 1960. Respiratory regulation in amphibian development. *Biol. Bull.*, **119**: 428–439.

Gregg, John R., 1962. Anaerobic glycolysis in amphibian development. *Biol. Bull.*, **134**: 555–561.

Gregg, John R., and Margit Kahlbrock, 1957. The effects of some developmental inhibitors on the phosphorus balance of amphibian gastrulae. *Biol. Bull.*, **113**: 376–381.

Gregg, John R., and Deana Klein, 1955. Morphogenetic movements of normal and gastrula-arrested hybrid amphibian tissues. *Biol. Bull.*, **109**: 265–270.

Gregg, John R., and Frances L. Ray, 1957. Respiration of homogenized embryos: *Rana pipiens* and *Rana pipiens* ♀ × *Rana sylvatica* ♂. *Biol. Bull.*, **113**: 382–387.

Harrison, Margaret N., 1963. Phosphorus metabolism in aerobic and anaerobic normal and hybrid frog embryos. Master's Thesis, Duke University.

Moore, J. A., 1946. Studies in the development of frog hybrids. I. Embryonic development in the cross *Rana pipiens* ♀ × *Rana sylvatica* ♂. *J. Exp. Zool.*, **101**: 173–220.

Ornstein, Norma, and John R. Gregg, 1952. Respiratory metabolism of amphibian gastrula explants. *Biol. Bull.*, **103**: 407–420.

Shumway, W., 1940. Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, **78**: 139–147.

Slein, M. W., G. T. Cori and C. F. Cori, 1950. A comparative study of hexokinase from yeast and animal tissues. *J. Biol. Chem.*, **186**: 763–780.

THE EFFECT OF NITROGEN, OXYGEN AND CARBON DIOXIDE IN PRODUCING THE DISTRESS SYNDROME IN TAPHIUS GLABRATUS (GASTROPODA, PULMONATA)

HAROLD W. HARRY AND JEROME B. SENTURIA[1]

Biology Department, Rice University, Houston 1, Texas

The fresh-water snail, *Taphius glabratus* (*Australorbis* or *Planorbina* of authors, but see Harry, 1962), shows a special reaction, the distress syndrome, in concentrations of toxic substances which are intermediate between those concentrations allowing normal behavior and those producing sustained retraction (Harry and Aldrich, 1963). The most obvious symptom of the distress syndrome is the continued extension of the cephalopedal mass beyond the shell's aperture, without the attachment of the foot. The snail is apparently relaxed, or paralyzed, and in more severe cases there is gross sloughing of the cells in the distal parts of the tentacles, with basal tentacular swelling. Certain heavy metals, notably zinc, cadmium and copper, were found to evoke the distress syndrome in lower concentrations than any other of the 22 ions tested. Since the manifestation of distress is grossly the same regardless of the kind of ion which produces it, it is logical to assume that distress may be caused by a single physiological mechanism.

Yager and Harry (1964) compared the uptake of radioactive zinc, copper and cadmium by this snail. They found that under comparable conditions the patterns of uptake were similar for all three metals. Snails showing normal behavior often took up more of the metals than snails which were in distress. It was concluded that distress was dependent upon the concentration of the noxious ion in the external medium rather than on the amount of the ion which entered the snail. They further suggested that these substances may produce distress by impairing the permeability of the surface membranes, and that the impairment might be only partial, but sufficient to impede the required rate of passage of materials which must pass (*e.g.*, oxygen and waste materials) in order for the snail to maintain normal behavior.

The present study was made to explore the latter hypothesis. Specifically, we wished to determine whether distress could be produced by subjecting the snails to anaerobic conditions, or high concentrations of carbon dioxide or oxygen in the environment, and to determine what behavioral responses would result when the snails were exposed to various combinations of nitrogen, carbon dioxide and oxygen.

We gratefully acknowledge the generosity of Dr. Clark P. Read, for making this study possible in his laboratory, and for critically reviewing the manuscript. Dr. Glenn W. Harrington rendered much helpful advice and assistance. The

---

[1] Present address: Department of Zoology, University of Texas, Austin, Texas.

study was supported in part by National Science Foundation grant GB 820 and U. S. Public Health Service Grant 5T1 AI 106-04.

MATERIALS AND METHODS

All snails were from an inbred laboratory stock originating in Puerto Rico. They were maintained in 50-liter metal-framed aquaria containing Houston tap water. The water was filtered initially through charcoal, and continuous charcoal filtration was provided by external aquaria filters. A few grams of chemically pure calcium carbonate were added weekly, and aeration was continuously provided. Fresh lettuce constituted the only food. No substrate material or aquatic plants were present in the aquaria, but small sheets of polyethylene plastic were introduced, as this is preferentially used by the snails for egg deposition.

Snails selected for each experiment were placed for 30 minutes in about 200 ml. of distilled water, which was changed once. They were then blotted on paper towels and all surface water was removed with soft absorbent paper tissue. Each snail was weighed to the nearest milligram. The total range of weights for all snails used was 218 to 474 mg., averaging 330.9 mg. The weight range of snails used in any single experiment varied between 55 and 177 mg., averaging 110 mg. New snails were used for each experiment.

In some preliminary experiments the control snails showed distress in distilled water which had been prepared from tap water passed through a large ion-exchange apparatus providing the bulk distilled water for the laboratory, then redistilled in a glass container having a chromium-plated metal heating element exposed to the water. Analysis of this water by the dithizone method of Huff, as modified by Lakin *et al.* (1952), showed an appreciable amount of undetermined heavy metals was present. Water for the definitive experiments was therefore prepared by passing the bulk demineralized water of the laboratory through Amberlite MB-1 ion exchange resin (Mallinckrodt Co.). Following the "general test procedure," as described by Lakin *et al.,* we found that 100 ml. of this water did not produce a grossly detectable color change in 1.0 ml. of 0.0016% dithizone solution. This water did not distress the snails.

All glassware was chemically cleaned by washing it in Alconox, followed by rinsing in dilute hydrochloric acid and several rinses of the water demineralized with Amberlite.

Flowing system experiments. This series of experiments was designed to test the effect of regulated amounts of the several gases on the snails when waste products are minimized by diluting them. Pyrex Erlenmeyer flasks of 250-ml. capacity were used for test and control animals. One hundred ml. of the demineralized water were put in each flask, each of which was stoppered with a two-holed rubber stopper. One hole served as the exhaust vent, and through the other the gas was introduced through a small glass tube with its free end drawn out into a capillary tip, inserted well below the surface of the water. Plastic tubing connected the glass tubes to a manifold with brass valves which allowed the gas to be regulated in each flask. The manifold was connected to a water bath through which the gases were bubbled (250 ml. of distilled water) directly from the pressure tanks. All gases were obtained from a local supplier of industrial gases, and were guaranteed to be 99.5% pure, or better. Only enough gas was introduced into the experimental flasks to keep the water gently agitated. After the water had been flushed with the test gas for 30 minutes or longer, one snail was introduced into each flask. Five snails were used in each experiment, and each experiment was duplicated at least once. Controls consisted of two flasks similarly prepared but with air introduced from an aquarium air pump. All exposures were at room temperature (22–24° C.).

Snails were exposed for 24 hours to the experimental gas. Any snails which crawled above the water during the experiment were put down again by gently tilting the flask. Observations were made about hourly at the beginning and end of each experiment, and at occasional periods between.

At the end of the exposure period, the pH of the water was determined with hydrogen ion test papers immediately after the gas was turned off. The water and snail of each flask were then gently poured into individual beakers for observation during the following 24 hours. A small piece of lettuce was provided each snail during this recovery period.

Closed system experiments. These two series of experiments were designed to determine whether distress or sustained retraction could be induced by the accumulation of waste products of the snail with concomitant depletion of the oxygen supply. (1) *Boiled water experiment.* Pyrex reagent bottles of 500- and 60-ml. capacity and weighing bottles with ground glass stoppers of 30-ml. capacity were washed as described above. These were filled with demineralized water and set in a pan of water on a hot plate and boiled for 30 minutes. They were then cooled to room temperature, and the snails, previously kept for 30 minutes in demineralized water, were added to each bottle. A small amount of boiled, cooled water was added to fill the bottle, and the glass stopper inserted carefully, to exclude all bubbles of air. This was easily achieved in the 60- and 500-ml. bottles. In the 30-ml. weighing bottles, owing to the concave bottom of the stoppers, a large bubble was unavoidable. No attempt was made to seal the stoppers with wax or grease. Five bottles of 500-ml. capacity each contained one snail. Four bottles of 60-ml. capacity each contained one snail, four each contained two, and four each contained three snails. The weighing bottles each had one snail. (2) *Water with air experiment.* Stender dishes of 8-, 25- and 50-ml. capacity were cleaned as described. Two milliliters of demineralized water were put in each of the smaller dishes, 5 ml. in each of the medium-sized ones and 10 ml. in each of the larger ones. After one snail (previously conditioned for 30 minutes in demineralized water) was put into each dish, the glass tops of the dishes were sealed in place with Lubriseal (stopcock grease, A. H. Thomas Co.). Five dishes of each size were used in the experiment.

RESULTS

The nature of the distress syndrome is such that it is only diagnosed with certainty after observing the snails over a period of several hours. This is particularly true if the distressed condition is mild. Snails attached but not crawling, or which crawled above the water, were considered normal in the present study. Those retracted just to the aperture of the shell or extended slightly beyond, but not attached, were considered to be in distress if they maintained this behavior for several hours. Only snails which remained retracted at least one-fourth whorl into the shell were diagnosed as "retracted," in the sense used by Harry and Aldrich (1963). In most cases the behavioral pattern exhibited at the end of 24 hours was assumed in one or a few hours after the experiment began, but in a few experiments the snail's reaction became more extreme during the experiment, passing from a normal to a distressed state, or from distress to sustained retraction.

In contrast to the ion-induced distress studied by Harry and Aldrich, where distressed snails were generally on the bottom of the bowl, most distressed snails in the present experiments floated at the water's surface.

The pH was 5.0 to 6.0 at the end of all experiments.

Flowing system experiments. The results of experiments in which gases were passed continuously through the experimental system are shown in Table I. The reaction of the snails was remarkably uniform in most experiments. In those few experiments in which more than one type of reaction was encountered, the number of snails showing a particular type is indicated as a prefix to the letter designating the reaction.

TABLE I

Response to various gas concentrations

% of gas			Reaction during	
N_2	CO_2	O_2	Experiment	Recovery
100			9N 1R	9N 1R
		100	10N	10N
95	5		10D	10N
80	20	–	6D 4R	10R
	100		10R	10R
75	5	20	10N	10N
76	20	4	10N	8N 2R

N—Normal; D—Distressed; R—Sustained retraction.
Reactions as recorded at end of 24 hours' exposure and 24-hour recovery period.

The one snail which showed sustained retraction during the experimental period when exposed to nitrogen continued this throughout the recovery period, and may be discounted as an anomaly. All snails exposed to pure oxygen showed less tendency to crawl out and more active movement than did those of any other experiments.

The snails exposed to 5% carbon dioxide and 95% nitrogen tended to remain attached but motionless during the first 12 hours of the exposure. All had their foot well extended but not attached during the last few hours of the experiment, but there was no obvious tentacular damage. During the recovery period this mild distress persisted for a few hours, but by 24 hours all had become attached to the substrate and had fed on the lettuce.

Snails exposed to 20% carbon dioxide and 80% nitrogen all showed very typical distress in the first few hours of the experiment, with marked tentacular sloughing and basal swelling. By the end of the experiment four were retracted. During the recovery period the remainder became retracted, and all were obviously dead, with the water showing a bacterial turbidity.

In 100% carbon dioxide all snails retracted about one-fourth whorl into the shell within the first 30 minutes of the experiment, and this retraction was sustained during the entire experimental and recovery periods. Bleeding was plainly evident in the bronzing of the water, and the snails were evidently dead by the end of the recovery period.

The mixtures of gases containing both oxygen and carbon dioxide were actually mixtures of air and carbon dioxide, but the minute quantities of other gases theoretically present (chiefly argon) are calculated as nitrogen in Table I. In those two experiments in which snails were exposed to 5% carbon dioxide, 20% oxygen and 75% nitrogen (CO_2 5%, air 95%), all snails were attached and frequently crawling during the exposure period, and all showed normal behavior and fed during the recovery period. The snails exposed to 20% carbon dioxide, 4% oxygen and 76% nitrogen (CO_2 20%, air 20%, nitrogen 60%) made repeated attempts to crawl out of the water, but none showed well-defined distress or sustained retraction during the experimental period. Most showed normal behavior during the recovery period, and the two which showed sustained retraction may be discounted as anomalous.

Closed system experiments. In the experiment in which snails were kept in closed bottles of boiled water without air, all were normal at the end of 60 hours. By 72 hours all were normal except for two in the 500-ml. bottles, one of which showed distress, while the other was retracted. All snails showed normal behavior in the experiments using Stender dishes throughout the 48 hours of the experimental period.

DISCUSSION

Von Brand and co-workers (von Brand, 1955; von Brand *et al.*, 1950, 1955; Mehlman and von Brand, 1951; von Brand and Mehlman, 1953; Newton and von Brand, 1955) demonstrated that *Taphius glabratus* can withstand anoxic conditions for many hours. After establishing that 95% of these snails survived for 16 hours when exposed to pure nitrogen, while only 25% survived at 24 hours in their experimental system, they used 16 hours as the period of exposure to nitrogen in subsequent studies on the anaerobic metabolism of this snail.

In our experiments with approximately 100% nitrogen the results were similar to those of the workers cited. Evidently the majority of these snails can tolerate anoxia induced by nitrogen for at least 24 hours. Nitrogen was not as debilitating as carbon dioxide.

However, whereas our snails showed essentially normal behavior in nitrogen throughout the experimental period, von Brand *et al.* (1950; pp. 276 *ff.*) reported a reaction which is distinctly and typically distress, in all 18 species of prosobranchs and pulmonates (including *T. glabratus*) which they exposed to pure nitrogen: "The behavior of the snails under anaerobic conditions was quite characteristic. All extended maximally out of their shells and soon, at least within a few hours, became completely motionless. If not used for chemical determinations, the snails, after the end of the anoxic period, were placed into beakers containing aerated dechlorinated tap water. As long as they were fully extended, these recovered completely, resuming motion soon after restoration of aerobic conditions. If the anaerobic period lasted too long, on the contrary, the snails began to hemorrhage and finally retracted into their shells. This seemingly indicates that the above-mentioned lack of motion was not a complete paralysis. Snails which had retracted into their shells during the anaerobic period did not recover during a subsequent aerobic period. Whether, in all cases, they actually died during the anaerobic period, or died shortly thereafter, could not be determined."

Any of several factors may account for the snail's more extreme reaction to nitrogen which von Brand *et al.* found. Our experimental system was one in which the container was continually flushed with gas, which would have carried away and minimized the concentration of volatile waste products of the snail. The system used by the workers cited was a closed system of small volume, which allowed waste products to accumulate, and to be concentrated in the immediate vicinity of the snail. The fluid phase of our system consisted of 100 ml. of water, whereas they used only 2 ml. Thus, any non-volatile waste products would be in dilutions 50 times greater in our experiments than in theirs, and presumably the toxicity of such materials would be lessened by a comparable amount.

The amount of oxygen originally present in our system may have been greater than that in theirs, owing to the larger volume of water, but the initial period of flushing with the test gas, and continuous flushing throughout the experiment would minimize this. The nitrogen we used was taken directly from the tank, with no further treatment except bubbling it through a water trap, whereas that used by von Brand *et al.* was further purified by being passed over hot copper. It may be argued that sufficient oxygen was present in our gas to support normal activity of the snails, though impurities were probably less than 0.05% in the gas we used.

Probably the most significant difference in the two experimental systems was their use of tap water, which, though dechlorinated, might yet have contained enough copper to have produced distress. Such an amount of copper is often present in tap water, and can originate from the copper fixtures under sinks. The amount of copper is likely to be high in tap waters low in total dissolved solids (less than 100 parts per million).

Although Newton and von Brand (1955) have reported differences between strains of this snail in their ability to withstand anoxia, it is unlikely that this could account for the greater tolerance of the Puerto Rican strain we used than they found in two strains from South America. Their experimental system was such that distress and more severe reactions might have been produced by factors other than anoxia *per se*.

As von Brand (1946; p. 91; 1950; p. 273, *q.v.* for references to several earlier studies) has noted, "the fact that aquatic snails possess a certain tolerance toward the lack of oxygen has been known for some time." Our experiments merely indicate that this tolerance may be greater than previously realized in the case of *T. glabratus*.

The snails which we exposed to oxygen showed no marked disability, and were indeed more active and content to remain below the water line than in any other experiments. *T. glabratus* is thus not among those animals to which higher than normal concentrations of oxygen are toxic (von Brand, 1946; Fox and Taylor, 1955). Fox and Taylor (1955) kept the closely related snail, *Planorbarius corneus*, for over a month in flasks of water through which 100% oxygen was bubbled. Although they concluded that the snails slowly succumbed during that time, from 21% to 80% of the snails survived after 35 and 37 days, respectively, in the several experiments they reported. They also noted that *Planorbarius* lived well for over a month in water through which a gas mixture of 4% oxygen and 96% nitrogen was continuously bubbled.

Von Brand and Mehlman (1953) have shown that the oxygen consumption by *T. glabratus* is directly proportional to the partial pressure of the oxygen in the experimental gas to which they are exposed, in mixtures of oxygen and nitrogen in which the oxygen varies from 5% to 100%. The amount of oxygen consumed depended on the temperature, nutritional state and whether the snails had been exposed to anoxia.

There seem to be no previous studies on the effect of varying carbon dioxide tensions on fresh water snails. The severity of the disability produced by this gas in the absence of oxygen seems to be directly proportional to the partial pressure of the carbon dioxide, or, by implication, the tension of the carbon dioxide in the aqueous phase of the experimental system. While the exact limits of the concentrations of this gas which will allow normal behavior, produce distress or sustained retraction were not determined, it is significant that distress typical of that produced by inorganic ions was produced by intermediate concentrations of carbon dioxide, in the absence of oxygen.

In the presence of oxygen, partial pressures of 5% and 20% carbon dioxide were much less toxic. Whatever the mechanism of toxicity of carbon dioxide may be, its effect can be diminished if oxygen is available. This is the more remarkable since even a 20% partial pressure of carbon dioxide, which is about 666 times as concentrated as the partial pressure would normally be in nature, was only mildly debilitating to the snails in the presence of 4% oxygen, which is about one-fifth as concentrated as it would normally be in nature. In so far as the gases involved in respiration may be limiting factors to the ecological distribution of this snail, it would seem that the presence of carbon dioxide, rather than the absence of oxygen, is the significant factor. More specifically, the presence of elevated carbon dioxide concentrations in the complete absence of oxygen (as in some underground waters) may be a limiting factor in nature.

In the experiments in which the snails were in closed bottles of boiled water without air, and in the water with air in the Stender dishes, the dilution of waste products even in 2 ml. water and 8 ml. air (roughly comparable to the 2 ml. water and 15 ml. air in the Warburg flasks used by von Brand and co-workers) evidently would be sufficient to render such wastes innocuous. These experiments also support the view that the quality of water, or some factor other than oxygen depletion and waste accumulation produced the distress reported by von Brand *et al.* in the anoxic experiments they devised.

Although the results reported above shed no light directly on the mechanism responsible for producing distress, they are compatible with the hypothesis that the failure of passage of materials at critical rates through surface membranes may be one factor in producing this phenomenon. It is generally assumed that the integument of fresh-water snails (Zaaijer and Wolverkamp, 1958; p. 67) as well as other organisms (Jacobs, 1920a, 1920b; Brandt, 1945) is very permeable to gases, but this does not exclude the possibility that there may be factors, includ-

[illegible] materials previously shown to produce distress, which can greatly alter the permeability of the membranes to gases. Brandt (1945; p. 32 *ff.*) states that carbon dioxide enters cells more rapidly than mineral acids, oxygen, nitrogen or other gases. He cites several references of more extensive studies on this aspect of the subject, but concludes, "it is at present unknown in what way carbon dioxide affects the state of the protoplasm and its function, except for its probable effect on pH." Jacobs (1920a, 1920b) had earlier argued that carbon dioxide probably has some specific effect on biological phenomena, quite apart from its ability to influence the pH.

Nothing is known of the ability of the blood of *Taphius glabratus* to transport carbon dioxide and oxygen. Zaaijer and Wolverkamp (1958) studied the hemoglobin-oxygen equilibrium in the blood of *Panorbarius corneus,* finding quite normal curves in the absence of carbon dioxide, when only temperature and the partial pressure of oxygen were varied. But when they introduced the additional factor of carbon dioxide and varied the pH in their pooled *in vitro* samples, they encountered "highly irregular and unpredictable" results, often including a negative Bohr effect when the pH was reduced below 8.0. They concluded that the blood buffering substances, proteins and salts of these snails may be variable. Targett (1963) recently showed that the blood proteins of *Taphius glabratus* may vary quantitatively and qualitatively, for causes yet unknown.

Zaaijer and Wolverkamp consider only the concentration of carbon dioxide in the external environment in seeking an explanation for its effect on hemoglobin, and they overlooked the possibility that the amount of carbon dioxide produced by the snail may be significant. Von Brand *et al.* realized the importance of the snail's own metabolism as a source of carbon dioxide, but point out in several papers that it is impossible at present to measure the internal concentration of this gas, owing to the interference produced by the snail's shell. To this we may add that the ubiquitous calcareous granules of the snail's connective tissue might also contribute to this difficulty, even if carbon dioxide production of snails with shell removed were to be studied. In any event, among numerous papers on snail respiration studies, no data on carbon dioxide production in aerobic conditions have been published, but its production in anaerobic conditions have been reported by Mehlman and von Brand (1951) and Newton and von Brand (1955).

SUMMARY

1. *Taphius glabratus* shows no abnormal reaction to anoxia induced by nitrogen during 24 hours' exposure. It also shows normal behavior in pure oxygen for that length of time.

2. In the absence of oxygen, 5% carbon dioxide produces mild distress, from which snails recover; 20% carbon dioxide evokes severe destress, from which snails did not recover; and 100% carbon dioxide resulted in immediate retraction and death.

3. In the presence of even smaller than normal amounts of oxygen, 5% and 20% carbon dioxide produced no deleterious effects on the snail.

LITERATURE CITED

Brandt, K. M., 1945. The metabolic effect and the binding of carbon dioxide in baker's yeast. *Acta Physiologica Scandinavica*, **10** : (Supp. 30) 206 pages.

Fox, H. M., and A. E. R. Taylor, 1955. The tolerance of oxygen by aquatic invertebrates. *Proc. Roy. Soc. London, Ser. B*, **143** : 214–225.

Harry, H. W., 1962. Critical catalogue of the nominal genera and species of neotropical Planorbidae. *Malacologia*, **1** : 33–53.

Harry, H. W., and D. V. Aldrich, 1963. The distress syndrome in *Taphius glabratus* (Say) as a reaction to toxic concentrations of inorganic ions. *Malacologia*, **1** : 283–289.

Jacobs, M. H., 1920a. To what extent are the physiological effects of carbon dioxide due to hydrogen ions? *Amer. J. Physiol.*, **51** : 321–331.

Jacobs, M. H., 1920b. The production of intracellular acidity by neutral and alkaline solutions containing carbon dioxide. *Amer. J. Physiol.*, **53** : 457–463.

Lakin, H. W., H. Almond and F. N. Ward, 1952. Compilation of field methods used in geochemical prospecting by the U. S. Geological Survey. *U. S. Geol. Survey Circular*, **161** : 1–34.

Mehlman, B., and T. von Brand, 1951. Further studies on the anaerobic metabolism of some freshwater snails. *Biol. Bull.*, **100** : 199–205.

Newton, W. L., and T. von Brand, 1955. Comparative physiological studies on two geographical strains of *Australorbis*. *Exp. Parasitology*, **4** : 244-255.

Targett, G. A. T., 1963. Electrophoresis of blood from intermediate and nonintermediate snail hosts of schistosomes. *Exp. Parasitology*, **14**: 143–151.

von Brand, T., 1946. Anaerobiosis in Invertebrates. Biodynamica, Normandy, Missouri. 328 pages.

von Brand, T., 1955. Anaerobiosis in *Australorbis glabratus*. Temperature effects and tissue hydration. *J. Washington Acad. Sci.*, **45** : 373-377.

von Brand, T., H. D. Baernstein and B. Mehlman, 1950. Studies on the anaerobic metabolism and the aerobic carbohydrate consumption of some freshwater snails. *Biol. Bull.*, **98** : 266–276.

von Brand, T., P. McMahon and M. O. Nolan, 1955. Observations on the postanaerobic metabolism of some freshwater snails. *Physiol. Zoöl.*, **28**: 35–40.

von Brand, T., and B. Mehlman, 1953. Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some freshwater snails. *Biol. Bull.*, **104**: 301–312.

Yager, C. M., and H. W. Harry, 1964. The uptake of radioactive zinc, cadmium and copper by the freshwater snail, *Taphius glabratus* (Say). *Malacologia*, **1**: 339–354.

Zaaijer, J. J. P., and H. P. Wolverkamp, 1958. Some experiments on the haemoglobin-oxygen equilibrium in the blood of the ramshorn (*Planorbarius corneus* L.). *Acta Physiol. Pharmacol. Neerlandica*, **7** : 56–77.

AN AUTORADIOGRAPHIC AND HISTOCHEMICAL INVESTIGATION OF THE GUT MUCOPOLYSACCHARIDES OF THE PURPLE SEA URCHIN (STRONGYLOCENTROTUS PURPURATUS)[1]

NICHOLAS D. HOLLAND[2] AND SISTER AQUINAS NIMITZ, O.P.[3]

Hopkins Marine Station of Stanford University, Pacific Grove, California

The older literature dealing with regular echinoid anatomy and histology mentions gland cells in the gut wall (see Hyman, 1955). More recently, Stott (1955) reported that gland cells in the wall of the sea urchin gut secrete an acid mucus, while Fuji (1961) reported that some sea urchin gut glands produce an acid mucus and others produce a neutral mucus. In the present study, some newer techniques of autoradiography and histochemistry have been applied to all regions of the gut of the purple sea urchin, *Strongylocentrotus purpuratus,* to reveal the distribution and characteristics of the mucopolysaccharides occurring in the wall of the digestive tract.

At present, the terminology for sea urchin gut regions is not entirely settled. It is, therefore, necessary to define the terms used in this paper. In *S. purpuratus,* the small buccal cavity extends from the mouth opening in the center of the peristome to the approximate level of the nerve ring. Interradially, each of the five teeth perforates the wall of the buccal cavity. In each radius is an outpocketing of the buccal cavity wall (Figs. 1 and 2). There are five such structures alternating with and lying between the five teeth. These outpocketings have not been named before, and we shall call them the radial buccal diverticula. In each interradius, there is an outpocketing of the roof of the buccal cavity (Figs. 1 and 5). Although these five outpocketings have been called the lips of the pharynx (Delage and Hérouard, 1903; Stott, 1955), we shall call them interradial buccal diverticula. The pharynx begins at the approximate level of the nerve ring and extends to the aboral surface of the lantern. The esophagus, which has about the same diameter as the pharynx, begins at the aboral surface of the lantern and extends to its junction with the stomach. This junction is conspicuous, since the stomach diameter is several times the esophagus diameter. Near the junction of esophagus and stomach, a slender tube, the siphon, leaves the main course of the gut to run parallel to the stomach. The stomach and siphon together make a nearly complete circuit of the body to the beginning of the intestine. The siphon rejoins the main part of the gut near the junction of the stomach and intestine. The intestine doubles back on the stomach and makes a nearly complete circuit of the body aboral to the stomach. The intestine ends indistinctly with a short rectum which ascends to the aboral pole, and terminates at the anus.

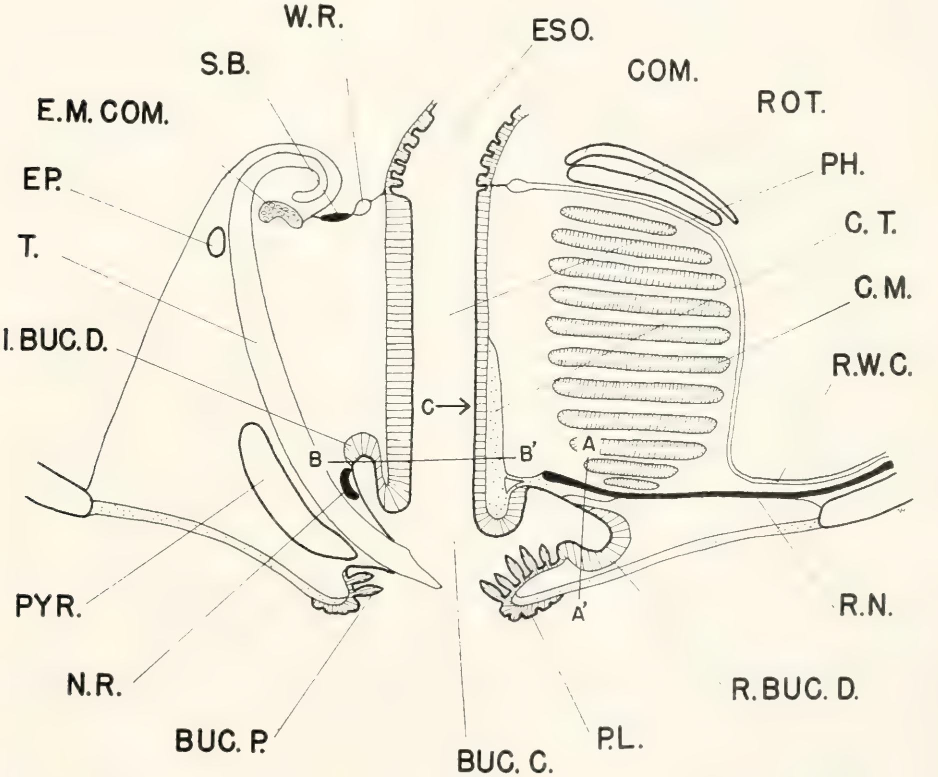
---

[1] This work was supported in part by USPHS Grant RG 4578 to A. C. Giese, and was performed during the tenure of predoctoral fellowships granted to the authors by the NSF and the USPHS, respectively. We are deeply indebted to Professor A. C. Giese, Professor L. R. Blinks, Professor D. P. Abbott, Dr. J. H. Phillips, Dr. S. Robrish, Mr. W. C. Austin, Mr. W. Lee and Mr. J. S. Pearse for providing facilities and for their invaluable advice and assistance during the preparation of this paper.

[2] Now at the Stazione Zoologica di Napoli, Naples, Italy.

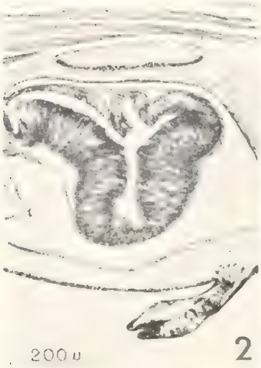
[3] Now at the Department of Biology, Dominican College of San Rafael, San Rafael, California.

Histologically, the entire digestive tract is composed of three layers. The inner lining is a tall columnar epithelium except in parts of the buccal cavity where it thins to a squamous epithelium. The layer covering the coelomic surface of the gut is a flagellated squamous epithelium, the visceral peritoneum. Between these two

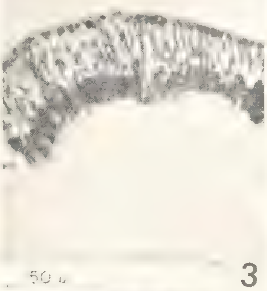


1

FIGURE 1. Vertical section through the Aristotle's lantern and adjacent structures of a 0.3-g. *S. purpuratus*. The right half of the figure is radial and the left half is interradial. The abbreviations, in clockwise order from the top of the figure, are as follows: ESO., esophagus; COM., compass; ROT., rotule; PH., pharynx, C. T., connective tissue; C. M., comminator muscle (these muscles are actually several times more numerous than depicted); R. W. C., radial water canal; R. N., radial nerve; R. BUC. D., radial buccal diverticulum; P. L., peristomial lip; BUC. C., buccal cavity; BUC. P., buccal papilla; N. R., nerve ring; PYR., pyramid; I. BUC. D., interradial buccal diverticulum; T., tooth; EP., epiphysis; E. M. COM., elevator muscle of compasses; S. B., spongy body; W. R., water ring. The haemal system is not shown in this figure.

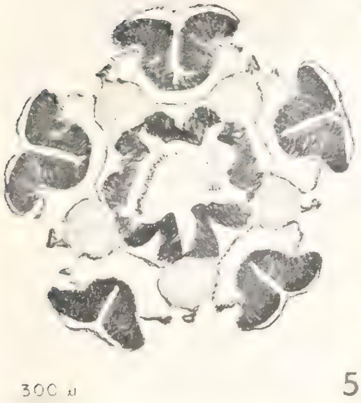


2



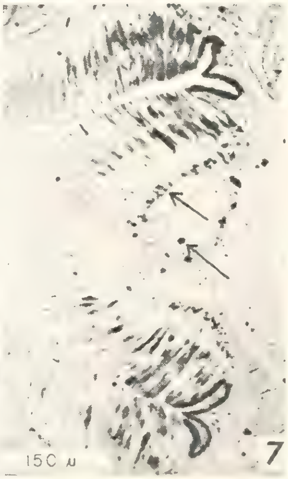
3

4

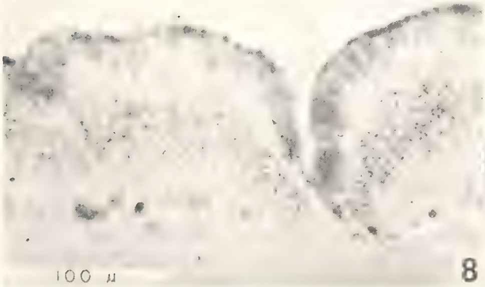


5

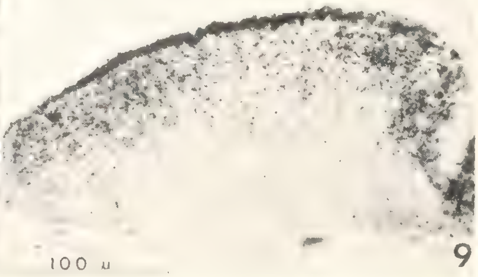
6



7



8



9

FIGURE 2. Cross-section of a radial buccal diverticulum and adjacent structures of a 0.3-g. urchin. The section is in the plane of A-A′ in Figure 1. From top to bottom are comminator muscles, radial nerve supported by connective tissue, radial buccal diverticulum surrounded by the peripharyngeal (or lantern) coelom, and peristomial membrane bearing a pedicellaria on its outer surface. Haematoxylin and eosin.

FIGURE 3. Vertical section of the peristomial lip of a 0.3-g. urchin. From top to bottom are the gland-containing epithelial layer facing the environment, the thick connective tissue-muscle layer, and the peritoneal layer bordering the peripharyngeal coelom. Alcian blue.

layers is a third consisting of connective tissue, nerves, and muscle fibers; this will be referred to as the connective tissue-muscle layer.

The physiology of digestion in *S. purpuratus* has recently been investigated by Farmanfarmaian and Phillips (1962). These workers found that the esophagus and first part of the stomach were the chief sites of digestion and absorption of C^{14}-labeled constituents of the alga, *Iridaea*.

MATERIALS AND METHODS

All the specimens of *Strongylocentrotus purpuratus* used were collected at low tide from tide pools near Yankee Point, California. Specimens not sacrificed fresh from the field were kept in containers of running sea water until used, and fed as much of the brown alga, *Macrocystis*, as they would eat.

Preliminary preparation of the tissues for histochemical and autoradiographic procedures was the same. Pea-sized urchins, averaging 0.3 g. fresh weight, were fixed whole in 50 ml. of sea water-Bouin's fluid. The sea water-Bouin's fluid decalcified the urchins completely in three days. Application of mild suction removed residual gas bubbles from the urchins after decalcification was complete. These specimens were then washed in water, dehydrated in ethanol, cleared in toluene, embedded in paraffin, and serially sectioned. Larger urchins, ranging from 5 to 20 g. (20 to 35 mm. in test diameter), were fixed in 150 ml. of sea water-

---

FIGURE 4. Vertical section of a buccal papilla of a 0.3-g. urchin. From top to bottom are the gland-containing epithelial layer bordering the buccal cavity, the connective tissue-muscle layer, and the peritoneal layer bordering the peripharyngeal coelom. Alcian blue.

FIGURE 5. Cross-section of the pharynx of a 0.3-g. urchin surrounded by all five interradial buccal diverticula. The section is in the plane of B-B′ in Figure 1. The spaces between the diverticula and pharynx are continuous with the peripharyngeal coelom, as can be seen from Figure 1. At this level the pharynx has a massive connective tissue support in each radial region. The pharyngeal lumen contains a bite of algae. Haematoxylin and eosin.

FIGURE 6. Cross-section of the pharynx of a 4.5-g. urchin. Only one complete interradial sector of tall epithelium and two radial grooves are shown; each radial groove is subdivided into several groovelets. At the left is the lumen of the pharynx, and next to this is the gland-containing epithelial layer. This epithelial layer is tall in the interradial areas and short in the radial grooves. To the right of the lining epithelium is the connectve tissue-muscle layer, which is thick interradially and thin radially. The peritoneal layer borders the peripharyngeal coelom at the right. Alcian blue.

FIGURE 7. Cross-section of the pharynx of a 4.5-g. urchin in the same orientation as Figure 6. In this Figure, the radial grooves are subdivided into two groovelets and the space between the lining epithelium and the connective tissue-muscle layer is an artifact. A number of coelomocytes may be seen wandering through the wall of the gut (arrows). Azure A.

FIGURE 8. Autoradiogram prepared from a cross-section of the esophagus of a 4.5-g. urchin killed one hour after injection of $Na_2S^{35}O_4$. The section has been stained through the emulsion with azure A. The lumen of the esophagus is at the top of the picture. The luminal border of the epithelium stains with azure A, showing beta metachromasia. A heavy concentration of silver grains lies over the middle third of the epithelial layer. At the base of the tall epithelium is a thin connective tissue-muscle layer in which several coelomocytes may be seen. Below the connective tissue-muscle layer is the peritoneal layer, which borders the perivisceral coelom.

FIGURE 9. Autoradiogram prepared from a cross-section of the esophagus of a 4.5-g. urchin killed 18 hours after injection of $Na_2S^{35}O_4$. The section has been stained through the emulsion with azure A. The orientation is the same as Figure 8, except the zone of silver grains lies over the luminal half of the epithelial layer.

Bouin's fluid, after 5- to 10-mm. diameter holes were opened on opposite sides of their tests at the ambitus to permit rapid entry of the fixative. After overnight fixation, the specimens were washed in water and dehydrated as far as 70% ethanol. At this point the urchins were dissected and segments of the following gut regions were removed for further processing: pharynx, esophagus, stomach with attached siphon, intestine, and rectum. The dehydration of the dissected tissues was then completed and they were subsequently cleared, embedded, and sectioned. For examination of the buccal cavity of larger urchins, the lantern and adjacent peristome were removed and processed as a unit like the small urchins.

Urchin gut regions, sectioned at 5 microns, were stained as follows:

(1) The periodic acid-Schiff (PAS) method for aqueous solutions was used according to the instructions of Humason (1962, p. 33). To remove any glycogen which might have survived histological processing, sections, prior to staining, were incubated in a 1% aqueous solution of diastase (malt, USP) for half an hour at room temperature (Pearse, 1960, p. 266).

(2) Alcian blue at pH 3 was used according to directions of Humason (1962, p. 269), omitting the counterstain. Some sections were stained sequentially with alcian blue and then PAS.

(3) Azure A (0.01%) was used according to instructions of Casselman (1959, p. 55). Some sections were stained sequentially with PAS and then azure A.

(4) The mercuric bromphenol blue method for aqueous solutions was used according to the directions of Mazia, Brewer and Alfert (1953).

(5) Sections adjacent to those used for histochemistry were stained routinely with haematoxylin and eosin.

For an autoradiographic investigation of the gut mucopolysaccharides, 100 μc. of $Na_2S^{35}O_4$ (specific activity = 142 millicuries per millimole) were obtained from New England Nuclear Corp., Boston. The S^{35}-sulfate came dissolved in slightly over 1 ml. of distilled water. The distilled water was evaporated by drying the solution for several hours in a 90° C. oven. The S^{35}-sulfate was then redissolved in 0.5 ml. of sea water, which closely approximates sea urchin coelomic fluid in composition. The sea water solution of S^{35}-sulfate was injected into the perivisceral coelom by way of the peristomial membrane. Four urchins (each with a fresh weight of 9 g.) each received 1 μc. of S^{35}-sulfate per gram. Two urchins (each with a fresh weight of 4.5 g.) each received 5 μc. of S^{35}-sulfate per gram. Injected urchins were returned to sea water until killed. Of the animals injected with 1 μc. per gram, one was killed in one hour, one was killed in 18 hours, and two were killed in 24 hours. Of the animals injected with 5 μc. per gram, one was killed in one hour and one was killed in 18 hours. These S^{35}-sulfate-treated urchins were then processed histologically. Sections of gut regions cut at 5 microns (as well as 5-micron control sections from urchins not treated with S^{35}-sulfate) were covered with Kodak AR-10 autoradiographic stripping film. Autoradiograms of guts of animals injected with 1 μc. per gram were exposed for 6 months, while autoradiograms of guts of animals injected with 5 μc. per gram were exposed for 47 days. Some autoradiograms were left unstained and mounted in the aqueous mounting medium described by Boyd (1955, p. 214) for phase contrast observation. Other autoradiograms were stained through the emulsion by the azure A procedure mentioned above and mounted in Permount.

RESULTS

Two of the mucopolysaccharides of the gut of *S. purpuratus* are widely distributed and need not be discussed separately for each gut region. The first of these mucopolysaccharides is contained in the spherules of certain coelomocytes wandering through the connective tissue-muscle layer and inner epithelium of the gut. These spherules stain orthochromatically with azure A, do not stain with PAS or alcian blue, and do not incorporate S^{35}-sulfate. A further discussion of these coelomocytes is beyond the scope of this paper. The second of the widely distributed mucopolysaccharides is found in the connective tissue-muscle layer of all gut regions. This layer stains with PAS after diastase digestion. The strength of the PAS reaction varies from urchin to urchin, ranging from weak to intense. The stronger the connective tissue-muscle layer stains with PAS, the stronger it stains with mercuric bromphenol blue. This layer does not stain with azure A or alcian blue and shows no uptake of S^{35}-sulfate in autoradiograms of S^{35}-sulfate-treated urchins. These results match the criteria defining neutral mucopolysaccharides in mammals (Spicer, 1963). The results with mercuric bromophenol blue suggest that this neutral mucopolysaccharide is associated with a protein, although the specificity of mercuric bromphenol blue for proteins has been questioned (Baker, 1958; Kanwar, 1960).

The histochemical tests demonstrate mucopolysaccharides in unicellular mucous glands in the inner epithelium of all gut regions preceding the junction of the esophagus and stomach. These mucous gland cells rarely exceed 6 microns at their point of greatest width, while their heights vary depending on their location and the size of the animal. The tallest mucous gland cells in a 0.3-g. urchin are 45 microns tall, while the tallest mucous gland cells in a 20-g. urchin are about 150 microns tall. These measurements demonstrate that the thickness of the gut wall relative to the weight of the animal is greater in small urchins than in large urchins. Some types of gland cells have mucopolysaccharides only in their luminal portions, and their unstained basal portions, while difficult to see in sections, presumably reach the base of the epithelial layer. The nucleus, when it can be seen, lies just basal to the secretion-swollen portion. The secretions of the mucous gland cells stain only weakly with mercuric bromphenol blue, and, therefore, contain comparatively little protein. The other properties of the mucous gland cells will be described for each gut region and are summarized in Table I.

The peristomial lip. The peristomial lip (or mouth rim), although not a part of the gut proper, is closely associated with it. The peristomial lip is characterized by a tall, glandular epithelium which distinguishes it from the rest of the peristomial membrane (Figs. 1 and 3). Each gland cell of the peristomial lip is as tall as the epithelium is thick. The basal half of each cell is swollen with a secretion, which reaches the exterior through the narrow neck of the cell. All peristomial lip mucous glands stain with PAS and with alcian blue. They do not stain with azure A and do not incorporate S^{35}-sulfate. The histochemical properties of this secretion do not correspond exactly to the properties of any known mammalian gut mucopolysaccharide (Spicer, 1963). However, it is probable that the peristomial lip gland cells contain a nonsulfated acid mucopolysaccharide containing some PAS-reactive residues or a mixture of neutral mucopolysaccharide and nonsulfated acid mucopolysaccharide components.

TABLE I

Summary giving the location in the gut and the nature of the secretion(s) for each type of mucous gland cell

Gut region	Location within gut region	Type of mucous gland cell	Nature of secretion(s) of mucous gland cell
Peristomial lip	Throughout	Type 1	Nonsulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
Buccal cavity	Buccal papillae	Type 1	Nonsulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
	Radial buccal epithelium	Type 1	Strongly acidic sulfated mucopolysaccharide
		Type 2	Sulfated acid mucopolysaccharide
Radial buccal diverticula	Throughout	Type 1	Nonsulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
		Type 2	Sulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
Interradial buccal diverticula	Throughout	Type 1	Nonsulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
		Type 2	Sulfated acid mucopolysaccharide
Pharynx	Radial grooves	Type 1	Strongly acidic sulfated mucopolysaccharide
	Interradial sectors	Type 1	Nonsulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
		Type 2	Sulfated acid mucopolysaccharide (and neutral mucopolysaccharide?)
Eosphagus	Throughout	Type 1	Strongly to moderately acidic sulfated mucopolysaccharide

The buccal cavity. Just within the mouth opening, the wall of the buccal cavity is thrown into numerous protuberances which we shall call buccal papillae. The gland cells in the epithelium covering each buccal papilla are not so abundant as the glands of the peristomial lip, and the secretion-containing part of the gland may occur at any level in the epithelial layer (Fig. 4). The secretion of the buccal papillae glands has the same properties and presumably the same composition as the secretion of the peristomial lip glands already described. In each interradial region just aboral to the buccal papillae, the buccal cavity lining thins to a squamous epithelium at the point where the tooth pierces it. In each radial region aboral to the buccal papillae, a tall epithelium lines the buccal cavity on either side of the opening to the radial buccal diverticulum. Here the buccal epithelium contains two kinds of mucous gland cells. The first type of gland cell predominates, crowding the epithelial layer from base to lumen. These cells show gamma metachromasia when stained with azure A, but do not stain with PAS or alcian blue. Autoradiograms show extensive uptake of S^{35}-sulfate has occurred in these glands in urchins killed 18 and 24 hours after injection of the radioisotope. These results match the criteria defining strongly acidic sulfated mucopolysaccharides in mammals (Spicer, 1963). The second type of mucous gland cell is less abundant than the first type, and its secretion-containing part is limited to the luminal third of the buccal epithelium. This second type of cell stains orthochromatically with azure A, but does not stain with PAS or alcian blue. Silver grains are located over this second type of gland cell in autoradiograms. Unfortunately, the proximity of this second cell type to the very radioactive first cell type, coupled with the low resolution of S^{35}-autoradiograms, makes it impossible to determine if any S^{35}-sulfate is incorporated by the second type of gland cell. However, in the light of results from other gut regions, these cells probably do incorporate moderate amounts of S^{35}-sulfate. The properties of the secretion of the gland cells of the second type are quite different from any known for mammalian gut mucopolysaccharides (Spicer, 1963). It is probable that the secretion is a sulfated acid mucopolysaccharide.

The radial buccal diverticula. The tall epithelium lining each radial buccal diverticulum contains two kinds of gland cell. The secretion-swollen part of the first kind of gland cell is located in the basal two-thirds of the epithelium and communicates with the lumen by way of a narrow neck. The secretion of these cells has the same properties and presumably the same composition as the secretion of the peristomial lip glands already mentioned. The secretion-filled portion of the less abundant, second type of gland cell is located in the luminal half of the inner epithelium of each radial buccal diverticulum. These cells stain orthochromatically with azure A, but do not stain with alcian blue. Some of these cells give a strong PAS reaction, while others give no PAS reaction. These cells incorporate moderate amounts of S^{35}-sulfate in urchins killed 18 or 24 hours after injection of the radioisotope. The orthochromatic material in these gland cells of the second type is probably a sulfated acid mucopolysaccharide. The PAS-positive material in some, but not all, of these cells could mean that the orthochromatic secretion is heterogeneous, some of it having many PAS-reactive residues and some of it having few PAS-reactive residues. On the other hand, the PAS could be staining a second type of secretion, a neutral mucopolysaccharide coexisting in some cells with the sulfated acid mucopolysaccharide.

The interradial diverticula. The tall inner epithelium of the interradial diverticula contains two types of gland cell. The secretion-swollen portion of the first type of gland cell is located in the basal one-fourth to one-half of the epithelium. Each of these gland cells communicates with the lumen *via* a narrow neck. The secretion of this first type of mucous gland cell has the same properties and presumably the same composition as the secretion of the peristomial lip glands already described. The secretion-swollen portion of the second type of gland cell occurs abundantly in the luminal one-half to three-fourths of the inner epithelium of the interradial buccal diverticula. The secretion of these cells stains orthochromatically with azure A, but does not stain with PAS or alcian blue. Autoradiograms prepared from sections of the interradial diverticula of urchins killed one hour after administration of S^{35}-sulfate show moderate uptake of the radioisotope by the middle third of the epithelial layer. At this level, the epithelial layer contains both the necks of the mucous gland cells of the first type and the most basal portions of the secretion-swollen parts of mucous gland cells of the second type. In the light of results reported below for the pharynx and esophagus, the radioactivity of the middle third of the epithelial layer is probably due to uptake of S^{35}-sulfate by the basal portions of the secretion-swollen parts of mucous gland cells of the second type. Autoradiograms prepared from sections of radial buccal diverticula of urchins killed 18 and 24 hours after injection of S^{35}-sulfate show a moderate to heavy concentration of the radioisotope throughout the luminal half of the epithelium. Thus, the second type of mucous gland cell in the interradial buccal diverticulum probably contains a sulfated acid mucopolysaccharide.

The pharynx. The pharynx has five longitudinal grooves, one in each radial region. The epithelial cells lining each pharyngeal groove are conspicuously shorter than the tall epithelial cells in each interradial sector of the pharynx. Each radial groove of the pharynx oral to arrow C in Figure 1 is usually single—this condition is shown in Figure 5. Each radial groove of the pharynx aboral to arrow C in Figure 1 is always subdivided into at least two groovelets (Fig. 7) and sometimes more than two groovelets (Fig. 6). The epithelium lining the radial grooves of the pharynx aboral to arrow C in Figure 1 is a continuous sheet of mucous gland cells. These gland cells are so closely packed that boundaries of individual cells are hard to see in sections. The portion of these mucous cells bordering the pharyngeal lumen is filled with a secretion which stains with azure A, showing gamma metachromasia (Fig. 7). This metachromatic secretion never stains with PAS, but sometimes stains weakly with alcian blue. Autoradiograms prepared from sections of pharynx from urchins killed one hour after injection of S^{35}-sulfate show moderate uptake of the radioisotope in the middle third of the radial groove cells. Autoradiograms prepared from sections of pharynx from urchins killed 18 or 24 hours after injection of S^{35}-sulfate show large amounts of the radioisotope localized in the luminal half of the radial groove cells. The properties of the metachromatic secretion of the radial grooves almost match the criteria defining strongly acidic sulfated mucopolysaccharides in mammals (Spicer, 1963). Only the tendency to stain with pH 3 alcian blue indicates that this sulfated mucopolysaccharide of the urchin may be a little less acidic than the mammalian strongly acidic sulfated mucopolysaccharides which Spicer's criteria define.

The tall epithelium of the interradial sectors of the pharynx contains two kinds of mucous gland cell. The first type of mucous gland cell is swollen at the level of the basal third of the tall epithelial layer and empties its secretion by way of a tenuous neck. Just before reaching the lumen, the diameter of the neck enlarges slightly, a feature which may be seen in Figure 6. The secretion of such a cell has the same properties and presumably the same composition as the secretion of the peristomial lip glands already described. The secretion-swollen part of the second type of mucous gland cell occurs in the luminal two-thirds of the tall interradial sectors of the pharynx. This second cell type is abundant only in those parts of the interradial sectors of tall epithelium near the radial grooves, as Figure 7 shows. The secretion of this cell type stains orthochromatically with azure A and stains strongly with PAS; it does not stain with alcian blue. Autoradiograms prepared from sections of pharynx from urchins killed 18 or 24 hours after injection of S^{35}-sulfate demonstrate a moderate amount of the radioisotope in areas of epithelium which contain this second type of cell. The second type of interradial pharyngeal gland cell is, therefore, comparable to some gland cells of the radial buccal diverticula. That is, each cell may contain a sulfated acid mucopolysaccharide rich in PAS-reactive residues, or it may contain sulfated acid mucopolysaccharide (perhaps similar to the sulfated acid mucopolysaccharide of the interradial buccal diverticula) plus a neutral mucopolysaccharide.

The esophagus. If a segment of living esophagus is turned inside out and viewed with a dissecting microscope, the exposed epithelial layer resembles the surface of a shucked ear of maize. Each area of epithelium resembling a kernel on the ear of maize corresponds to the tall epithelium seen in histological sections of the esophagus. The narrow channels which delimit the "kernels" correspond to the grooves seen in histological sections of the esophagus. Figure 8 shows two areas of tall epithelium separated by an esophageal groove. Since the connective tissue-muscle layer at the base of the tall epithelium is as thin as the connective tissue-muscle layer at the base of the short, groove-lining epithelium, the esophageal grooves are due entirely to differences in the height of the epithelial layer. There is only one kind of esophageal mucous gland cell, which is abundant in the grooves and in the tall epithelium. Azure A always stains the luminal portion of the esophageal mucous gland cells. The width of the azurophilic zone varies from urchin to urchin (Figs. 8 and 9), although, in a given individual, the width of the zone is relatively constant throughout the course of the esophagus. The azurophilic secretion usually shows gamma to beta metachromasia, but in some individuals it may show orthochromasia. In general, the wider the zone that stains with azure A, the greater its tendency to stain orthochromatically. The azurophilic secretion of the esophageal mucous gland cells never stains with PAS, but usually stains weakly with alcian blue. Autoradiograms of sections of the esophagus of urchins killed one hour after injection of S^{35}-sulfate show moderate uptake of the radioisotope by the middle third of the epithelial layer (Fig. 8). Autoradiograms of sections of the esophagus of urchins killed 18 or 24 hours after injection of S^{35}-sulfate show a heavy concentration of the radioisotope in the luminal half of the epithelium (Fig. 9). The properties of the azurophilic secretion in the luminal part of the esophageal gland cells indicate that it is a sulfated acid mucopolysaccharide. The acidity of the secretion appears to vary from urchin to urchin. In some urchins the acid is strong (comparable in strength to the radial groove secretion of the pharynx), but the acid may be of intermediate strength in those urchins which have an esophageal secretion staining orthochromatically with azure A.

The stomach, siphon, intestine and rectum. In none of these urchin gut regions does the inner epithelium contain unicellular gland cells filled with mucopolysaccharides. In some urchins, the luminal border of these gut regions may stain weakly with alcian blue. The source of this apparent acid mucopolysaccharide is unclear. It could be synthesized locally by epithelial cells which are not recognizably differentiated mucous gland cells. On the other hand, this acid mucopolysaccharide could well be produced by mucous gland cells in gut regions preceding the junction of the esophagus and the stomach, and then be carried into the gut regions following this junction to coat the inside wall of the gut.

DISCUSSION

In this paper it has been assumed that S^{35}-sulfate is incorporated only into sulfated mucopolysaccharides and not into proteins. Sulfated mucopolysaccharides and proteins are the only sulfur-containing molecules synthesized by animals and known to survive fixation and subsequent histological processing. The possible incorporation of S^{35}-sulfate into sulfur-containing amino acids, and subsequent use of such amino acids to synthesize proteins is considered very improbable for several reasons. Prosser and Brown (1961, p. 86), after a survey of the amino acid requirements of animals, conclude: "The most striking feature is the similarity in the [amino acid] requirements of all animals. Apparently the general pattern of loss of synthetic capacity of some nine (or ten) amino acids was established very early in animal evolution." Although amino acid requirements are not known for any echinoderm, it is most unlikely that they deviate from the clear-cut pattern seen in other animals. One of the essential amino acids which no animal is known to synthesize is the sulfur-containing amino acid, methionine. Animals can convert methionine into the sulfur-containig amino acids, cysteine and cystine, by a series of irreversible reactions. These three amino acids are the only sulfur-containing amino acids known to be involved in protein synthesis; none of them can be synthesized from sulfate by animals. Moreover, S^{35}-sulfate injected into the sea urchin fails to label the lantern, body wall, axial organ or gonads (which were unripe or immature in the urchins injected). It is especially important to note that the soft, aboral growing tips of the teeth do not label with S^{35}-sulfate. These tooth regions are the sites of intense cell proliferation, and should have a high rate of protein synthesis. If S^{35}-sulfate could be incorporated into proteins by the sea urchin, it should be incorporated wherever proteins are being synthesized, and not exclusively incorporated by regions of gut which contain mucopolysaccharides.

The mucous secretions of the gut of *Strongylocentrotus purpuratus* have the same general features as the gut mucous secretions of *Echinus esculentus* (Stott, 1955) and *Strongylocentrotus intermedius* (Fuji, 1961). In all these regular echinoids, the gut mucous secretions, most of them acidic, are produced by unicellular gland cells of the inner epithelium of gut regions preceding the junction of the esophagus and the stomach; following this junction, there are no mucous gland cells. Stott stained *Echinus* mucous gland cells with a haematoxylin and with mucicarmine. These stains unfortunately lack histochemical specificity. Fuji, however, used several good histochemical tests in his study of *Strongylocentrotus intermedius*. He stained gut mucopolysaccharides with PAS after salivary digestion (which is comparable to diastase digestion), and with toluidine blue (which is comparable to azure A). Fuji, who did not investigate the buccal cavity, claimed that both the esophagus and the radial grooves of the pharynx were crowded with gland cells containing a neutral mucopolysaccharide. In *Strongylocentrotus purpuratus,* the mucous gland cells of the esophagus and those of the radial groove of the pharynx contain no detectable neutral mucopolysaccharide. Therefore, there are interspecific differences in the details of mucous gland cell distribution and content in regular echinoid guts.

Table I shows that the radial buccal epithelium, the radial buccal diverticula, the interradial buccal diverticula and the interradial sectors of the pharynx each contains two kinds of gland cell. These gland cells were treated as two distinct cell types to facilitate exposition in the results section of this paper. However, it is possible that, in each gut region mentioned, the two mucous gland cell types are actually two successive stages in the secretory cycle of one type of gland cell. Anderson (1960, p. 381) has previously raised the possibility that morphologically different mucous gland cells in one region of an asteroid gut may represent different secretory phases of a single population of gland cells. In the case of the sea urchin gut, we have, at present, no convincing evidence for or against such a secretory cycle.

Sulfated acid mucopolysaccharides of the mammalian gut stain metachromatically with azure A, and nonsulfated acid mucopolysaccharides stain orthochromatically with azure A (Spicer, 1963). Sea urchin gut mucopolysaccharides differ markedly from mammalian gut mucopolysaccharides in their reactions to azure A. Nonsulfated acid mucopolysaccharides of the sea urchin gut do not stain at all with azure A, while sulfated acid mucopolysaccharides of the sea urchin gut may stain either metachromatically or orthochromatically with azure A. In the urchin gut, there is incorporation of S^{35}-sulfate wherever there is azurophilia of any kind. Apparently, in the urchin, a molecule of the orthochromatic sulfated mucopolysaccharide may incorporate detectable amounts of S^{35}-sulfate as sulfate esters without creating enough free electronegative surface charges to cause metachromasia. This might be due to a steric configuration that occludes the sulfate groups.

Mucopolysaccharide biosynthesis probably occurs by a complex series of steps. In the case of sulfated mucopolysaccharides, it is still not known whether the sulfation step occurs before or after polymerization of the sugars. It is known, however, that a compound rich in S^{35} appears in the vesicles of the Golgi complex of mammalian chondrocytes within three minutes of injection of S^{35}-sulfate (Porter, 1964). The S^{35}-sulfated sugars of such a compound would have to be either polymerized or protein-bound in order to survive fixation. The Golgi-associated vesicles might be the actual site of sulfation or they might be a center for rapid concentration or assembly of material sulfated elsewhere in the cell. With the passage of time, the S^{35}-sulfated material is detected in larger vesicles and finally is secreted from these vesicles into the extracellular environment (Porter, 1964). A similar type of secretory activity has been demonstrated for the Golgi apparatus of plant cells (Mollenhauer and Whaley, 1963). Here, secretion vesicles form at the edges of the stacked cisternae. These secretion vesicles pinch off from the cisternae, and become larger and more electron-dense as they move through the cytoplasm to the plasma membrane where their contents are secreted from the cell. The Golgi apparatus is further implicated in secretion by the findings of Hollman (1963), who investigated the ultrastructure of goblet cells, mucous gland cells of the rat intestine. He concluded (p. 547) that "there seems to be no doubt that the Golgi apparatus plays a predominant role in the secretion process of the goblet cell." In at least some of the mucous glands of the sea urchin's gut, the Golgi apparatus may play an important part in the secretion process. Mucous gland cells in several gut regions of the urchin show initial uptake of S^{35}-sulfate in the middle third of the cell (Fig. 8), and a subsequent migration of the label to the luminal part of the cell (Fig. 9). Therefore, either the sulfation step in the synthesis of some sulfated acid mucopolysaccharides or a concentration of sulfated products occurs in the gland cell region presumably containing the Golgi apparatus.

Mucous gland cells of the esophagus and of the pharyngeal radial grooves are refractory to specific histochemical tests for sulfated acid mucopolysaccharides in the region of initial S^{35}-sulfate uptake. In 18 hours, the sulfated material has migrated to the luminal parts of the cells and stains positively for sulfated acid mucopolysaccharides. A similar phenomenon was described by Immers (1961) for mucopolysaccharides in eggs and embryos of the sea urchin, *Paracentrotus lividus*. Immers claimed that these sulfated acid mucopolysaccharides were masked (*i.e.,* refractory to specific histochemical tests) when combined with protein, and that they became unmasked and stainable when split from the protein; French and Benditt (1953) proposed a molecular mechanism for protein masking of mucopolysaccharides. On the other hand, the region of initial sulfate uptake could be refractory to specific histochemical tests for sulfated acid mucopolysaccharides simply because the sulfated molecules are present in very small amounts, which are detectable autoradiographically, but not histochemically. If we accept the explanation of Immers, we may speculate that, in the sea urchin, sulfated acid mucopolysaccharides of some gland cells may be elaborated (or concentrated) in the Golgi apparatus in combination with a protein. The protein-mucopolysaccharide complex may subsequently migrate (perhaps *via* secretion vesicles) to the luminal part of the cell, where it dissociates, unmasking the sulfated acid mucopolysaccharide.

In *S. purpuratus* the mucous gland cells of the gut have several probable functions. These cells produce mucopolysaccharides which may lubricate the inner wall of the relatively narrow pharynx and esophagus. Such lubrication would protect the delicate gut wall during the passage of hard, sharp objects ingested, such as bites of encrusting coralline algae. Furthermore, the mucopolysaccharides produced in the buccal region of the urchin may well play an important part during ingestion of friable food material. When the teeth bite into a brittle object like a coralline alga, small particles, otherwise lost to the urchin, may stick to mucous secretions surrounding the teeth and thus get drawn into the buccal cavity.

Summary

1. A neutral mucopolysaccharide, probably associated with a protein, occurs in the connective tissue-muscle layer of all gut regions of the purple sea urchin.

2. In the inner epithelium, mucopolysaccharides are found in unicellular glands located in all gut regions preceding the junction of the esophagus and stomach. Such glands never occur in gut regions following this junction.

3. Many, and perhaps all, of the mucopolysaccharides of these unicellular glands are acidic. Of these acid mucopolysaccharides, some are sulfated and others are not.

4. Autoradiograms show that some gland cells which contain acidic sulfated mucopolysaccharides first incorporate S^{35}-sulfate in the middle third of the cell. In some cases, the initially-sulfated material is refractory to specific histochemical tests for sulfated acid mucopolysaccharides, perhaps because the mucopolysaccharides are masked by combination with protein.

5. Autoradiograms show a migration of sulfated material from the middle third to the luminal portion of some gland cells. In cases where the sulfated material was masked when synthesized in the middle third of the cell, it becomes unmasked when it reaches the luminal part of the cell.

LITERATURE CITED

Anderson, J. M., 1960. Histological studies on the digestive system of a starfish, *Henricia*, with notes on Tiedemann's pouches in starfishes. *Biol. Bull.*, **119**: 371–398.

Baker, J. R., 1958. Note on the use of bromphenol blue for histochemical recognition of protein. *Quart. J. Micr. Sci.*, **99**: 459–460.

Boyd, G. A., 1955. Autoradiography in Biology and Medicine. Academic Press, New York.

Casselman, W. G. B., 1959. Histochemical Technique. Methuen, London.

Delage, Y., and E. Hérouard, 1903. Traité de Zoologie Concrète. Tome III. Les Échinodermes. Schleicher, Paris.

Farmanfarmaian, A., and J. H. Phillips, 1962. Digestion, storage, and translocation of nutrients in the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **123**: 105–120.

French, J. E., and E. P. Benditt, 1953. The histochemistry of connective tissue: II. The effect of proteins on the selective staining of mucopolysaccharides by basic dyes. *J. Histochem. Cytochem.*, **1**: 321–325.

Fuji, A., 1961. Studies on the biology of the sea urchin. IV. Histological observation of the food canal of *Strongylocentrotus intermedius*. *Bull. Fac. Fish. Hokkaido*, **11**: 195–202.

Hollmann, K. H., 1963. The fine structure of the goblet cells of the rat intestine. *Ann. N. Y. Acad. Sci.*, **106**: 545–554.

Humason, G. L., 1962. Animal Tissue Techniques. Freeman, San Francisco.

Hyman, L. H., 1955. The Invertebrates. IV. Echinodermata. McGraw-Hill Book Co., Inc., New York.

Immers, J., 1961. Comparative study of the localization of incorporated ^{14}C-labeled amino acids and $^{35}SO_4$ in the sea urchin ovary, egg, and embryo. *Exp. Cell Res.*, **24**: 356–378.

Kanwar, K. C., 1960. Note on the specificity of mercuric bromphenol blue for the cytochemical detection of proteins. *Experientia*, **16**: 355.

Mazia, D., P. A. Brewer and M. Alfert, 1953. The cytochemical staining and measurement of protein with mercuric bromphenol blue. *Biol. Bull.*, **104**: 57–67.

Mollenhauer, H. H., and W. G. Whaley, 1963. An observation of the functioning of the Golgi apparatus. *J. Cell Biol.*, **17**: 222–225.

Pearse, A. G. E., 1960. Histochemistry, Theoretical and Applied. Churchill, London.

Porter, K. R., 1964. Cell fine structure and biosynthesis of intercellular macromolecules. *Biophys. J.*, **4**: 167–196.

Prosser, C. L., and F. A. Brown, Jr., 1961. Comparative Animal Physiology. Saunders, Philadelphia.

Spicer, S. S., 1963. Histochemical differentiation of mammalian mucopolysaccharides. *Ann. N. Y. Acad. Sci.*, **106**: 379–388.

Stott, F. C., 1955. The food canal of the sea-urchin *Echinus esculentus* L. and its functions. *Proc. Zool. Soc. London*, **125**: 63–86.

REPRODUCTION AND LARVAL DEVELOPMENT OF ACMAEA TESTUDINALIS (MÜLLER)[1]

MARGARET M. KESSEL

Department of Zoology and Entomology, University of Connecticut, Storrs, Connecticut

Although the biology and anatomy of *Acmaea testudinalis* have been studied (Willcox, 1900a, 1905, 1906) its larval development has never been described. Isolated stages of this limpet have been studied by Morse (1910), Thompson (1912), Thorson (1946), and Raven (1958). Boutan (1898, 1899), with *Acmaea virginea*, and Thorson (1935), with *Acmaea rubella*, have been the only workers to present any details of larval development in this genus. This report describes and illustrates the developmental stages of *A. testudinalis*.

The tortoise-shell limpet is a gastropod of the order Diotocardia, superfamily Patellacea and family Acmaeidae (Fretter and Graham, 1962). It is found on rocks and shells just below the low tide line on the coasts of islands and continents bordering the North Atlantic and also around the Arctic Circle to the Bering Sea (Grant, 1937).

Great variation in reproductive methods exists in the genus *Acmaea*. *A. testudinalis* is unisexual and lays its eggs in a thin mucous sheet. *A. virginea,* also unisexual, lays its eggs singly (Boutan, 1898). *A. fragilis,* a protandric hermaphrodite for a short time, also lays its eggs singly (Willcox, 1898, 1900b). *A. rubella* is a viviparous hermaphrodite (Thorson, 1935). Fertilization is external in *A. testudinalis* and *A. virginea*, though Willcox (1900b) mentioned that the renal papilla of *A. fragilis* might act as an intromittant organ.

An exhaustive study of stimuli affecting reproductive cycles and the relation of these cycles to geographic location and habitat of 21 species and subspecies of *Acmaea* on the Pacific Coast was made recently by Fritchman (1961a, 1961b, 1961c, 1962). He found that air and water temperatures, geographic range, phase of the moon and tidal cycles all could influence gonad development and spawning. For instance, *A. insessa* spawned at any provocation; *A. fenestrata cribraria* spawned three times a year; *A. persona* spawned once a year from March to April. *Acmaea scutum*, the Pacific limpet so closely related to *A. testudinalis*, has several partial spawnings a year and at no time is the gonad indeterminant. Fritchman (1961b) feels that these spawnings may have some relation to the full moon and its tides.

Breeding in the British limpet, *Patella vulgata*, was studied by Orton, Southward and Dodd (1956). Both *P. vulgata* and *P. coerulea* are protandric hermaphrodites (Fretter and Graham, 1962) but *P. depressa* is unisexual (Orton and Southward, 1961). All three lay their eggs singly.

---

[1] Contribution No. 30, Marine Research Laboratory, Noank. This work was part of a thesis presented to the Faculty of the Graduate School of the University of Connecticut for the degree of Master of Science.

Successful fertilization and rearing of larvae has been a problem confronting many workers who have investigated limpet development, as well as that of other organisms. Patten (1885) and later LoBianco in 1889 (Dodd, 1957) were successful in obtaining artificial fertilization with *Patella coerulea*. Unfortunately, Patten (1886), in studying the embryology of this species, was largely working with abnormal larvae (Dodd, 1957). However, Patten remained the primary source of information on limpet development until Smith (1935) published his work on development of *Patella vulgata*. Smith was able to raise a few to metamorphosis. Dodd (1951, 1957) has been successful in rearing large numbers of *P. vulgata* larvae through metamorphosis. Boutan (1899) raised larvae of *A. virginea* in tanks with sand-filtered running sea water, using algae, which came through the system, as food. Dodd's (1957) rearing method emphasized the importance of mechanical stirring of rearing jars from fertilization until the larvae began to settle.

MATERIALS AND METHODS

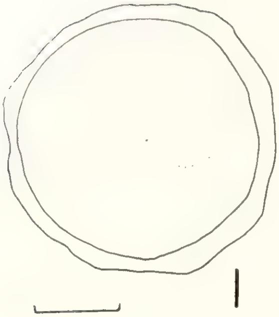
Adult limpets were collected at Noank, Connecticut, in June, July and December of 1962 and June of 1963, and at Watch Hill, Rhode Island, in May, July and August of 1962 and February, April and June of 1963. No extensive plankton samples were taken. Adults were kept in aquaria with running sea water in the Marine Research Laboratory at Noank and in the aquatic room of the Life Science building at Storrs. Microscopic algae on small rocks were provided for food.

Method of obtaining gametes

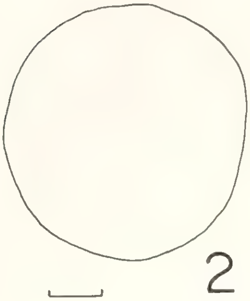
Ripe individuals were selected by observing the sole of the limpet's foot. Ripe males have a creamy streak down the underside of the foot, while females show a reddish area. This can best be seen through the glass side of an aquarium.

Two to four males and five to eight females were placed in acid-washed finger bowls (one for males and one for females) and brought out of the aquatic room where the water was 12.1 ± 0.5° C., into room temperature and allowed to warm slowly. Spawning would occur when water reached 19.5° to 22.5° C. Limpets also responded to gentle agitation of the water or small squirts of water into the nuchal cavity at those temperatures. By having males and females releasing gametes in separate bowls, polyspermy can be prevented.

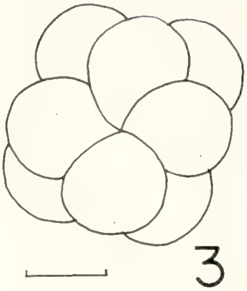
A tremendous amount of sperm is released, and is emitted for a longer period than the females' spawn. Each sperm is about 0.054 mm. long. Sperm are extremely active and remain viable for at least three hours. Eggs of *Acmaea testudinalis* are small (0.14 mm. in diameter) and tan to brick red in color. An egg taken from the ovary (Fig. 1) possesses an outer membrane which appears to swell on contact with water but later gradually disappears (Fig. 2). Unfertilized eggs fixed immediately after spawning show the eggs held in a sheet at regular intervals by their gelatinous coverings. Not all eggs are attached; some become free upon contact with the water. Therefore, it would appear that the thin mucous sheet is not formed by the sole of the foot, as Willcox (1905) suggested, but by agglutination of the gelatinous membranes about each egg.



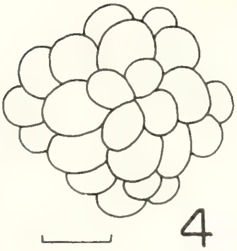
1



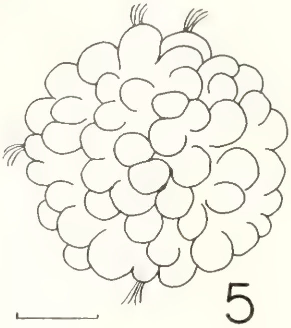
2



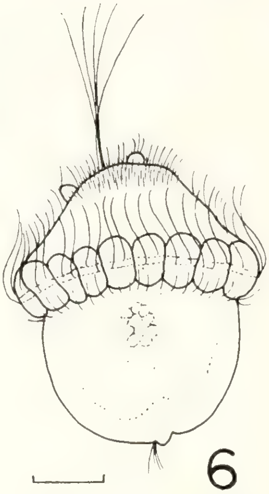
3



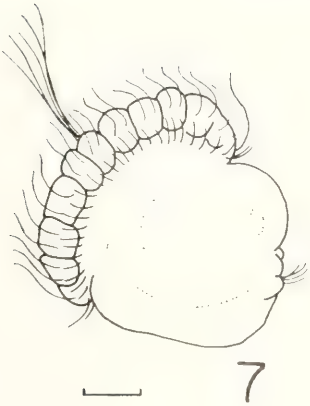
4



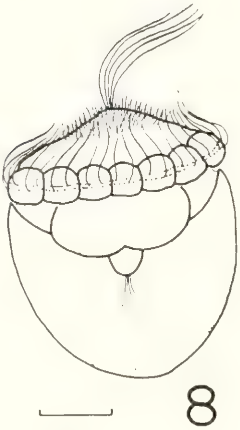
5



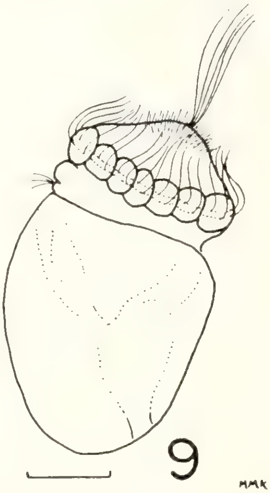
6



7



8



9

A camera lucida attached to a Wild M20 phase contrast microscope with a 20 × Fluotar objective and 10 × ocular were used for drawings. Figures 14 and 18 were sketched without the camera lucida because the larvae were too active at the time to use this attachment. For clarity, the number of cilia shown is less than in the living specimen. Each scale line in the figures equals 0.05 mm. except in Figure 18 where it equals 0.1 mm.

When the animal is releasing gametes, the shell is lifted high off the substrate and tilted toward the posterior end. In the male, sperm is squirted from the renal papilla; in the female, eggs seem to be ejected from the nuchal cavity. Usually eggs remain unfertilized or cleave abnormally if gonads are removed from the animals, as Dodd (1957) did with *Patella*. Best results were obtained when the limpets were allowed to spawn naturally in separate bowls.

Method for rearing larvae

Adults were taken out of spawning bowls and the eggs then poured through cheesecloth to remove any debris or fecal matter. The eggs were separated into four or five dishes, depending upon the number released. Larvae developed better in numbers (at least 400 eggs per dish) than with too few per container. Two to four drops of sperm suspension were placed in each egg dish and stirred. The rearing temperature was 12.1 ±0.5° C.

Trochophores developed within 10 to 13 hours. Since they swam at the surface of the water, the top portion of the water could be poured into clean bowls and aerated filtered sea water added. This eliminated uncleaved and abnormal eggs which remained on the bottom of the dish.

Water to be used was kept in a plastic jug and aerated continuously with an air hose inserted through a hole in the cap of the jug. It was found harmful to larvae to aerate rearing bowls. Water was changed every other day by pouring larvae and water through plankton netting with small enough mesh to prevent loss of larvae. In this case, #12 plankton netting, with a mesh opening of 110 microns, was used. Larvae were rinsed quickly into a clean bowl with clean water. Each fingerbowl was filled with about 200 ml. of water. This manner of water-changing was carried out until the larvae began to settle. At that time, water was pipetted off and clean aerated water added to the same dish. If the ciliate population had increased, the healthy larvae were removed with a pipette to clean dishes.

Feeding began at about 60 hours. A few drops of a culture of the diatom, *Phaeodactylum* sp., were added every other day until the larvae began to creep, at which time a bottom food was required. This was provided by placing a piece of *Ulva* (*Cytosiphon* was also recommended by Dodd, 1957) in an acid-washed finger bowl with aerated filtered sea water. Within a few days, spores were deposited on the bottom and the *Ulva* removed. Other algae such as diatoms may also be deposited there. Creeping larvae were transferred to the prepared dish and each individual placed in a cleared area. Larvae could then obtain their food by scraping

---

FIGURE 1. Egg taken directly from ovary; note sheath.

FIGURE 2. Fertilized egg; note absence of sheath.

FIGURE 3. Eight-celled stage; two hours 50 minutes after fertilization.

FIGURE 4. Multicelled stage; note micromeres; view of animal pole; four hours 40 minutes after fertilization.

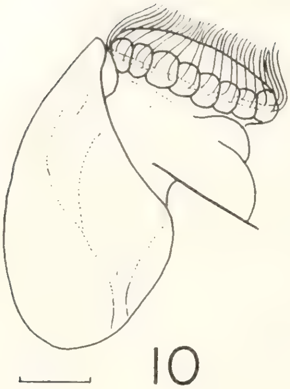
FIGURE 5. Cilia forming; nine and one-half hours after fertilization.

FIGURE 6. Ventral view of trochophore with stomodaeum; 25 hours after fertilization.

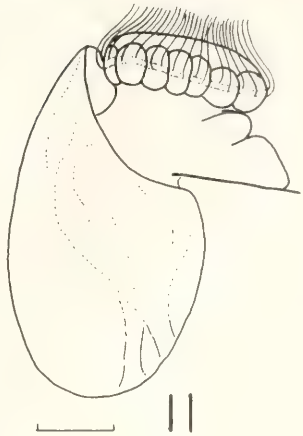
FIGURE 7. Ventro-lateral view of late trochophore; 30½ hours after fertilization.

FIGURE 8. Ventral view of early veliger; note position of foot and telotroch; 36 hours after fertilization.

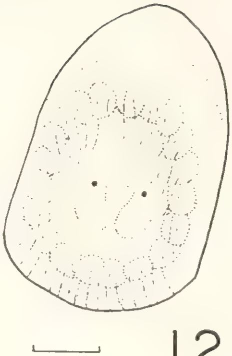
FIGURE 9. Lateral view of veliger; note position of telotroch; 47 hours after fertilization.



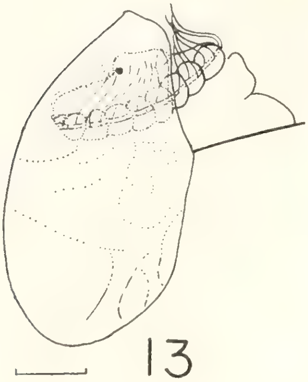
10



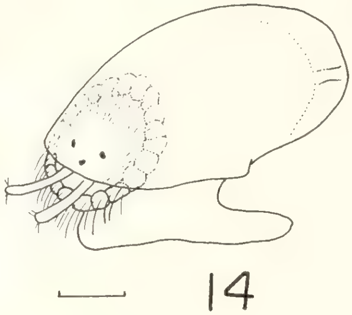
11



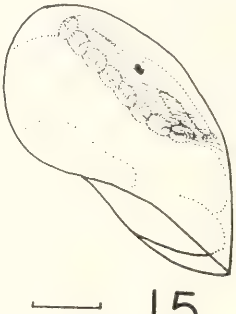
12



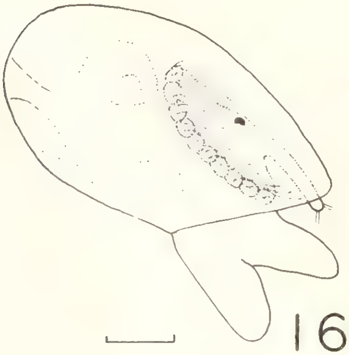
13



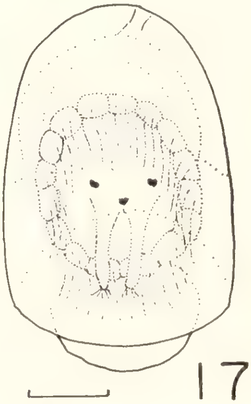
14



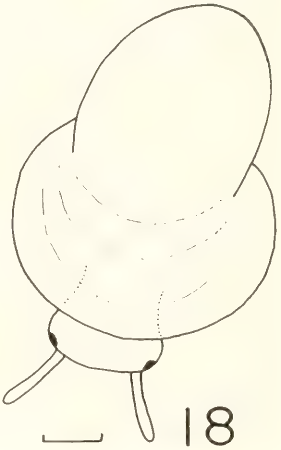
15



16



17



18

FIGURE 10. Lateral view of veliger after torsion; note position of muscle and foot; 62½ hours after fertilization.

FIGURE 11. Lateral view, note presence of second retractor muscle; 65 hours after fertilization.

FIGURE 12. Dorsal view of larva withdrawn into shell; note eyespots and tentacle rudiments; 85½ hours after fertilization.

FIGURE 13. Lateral view; 97 hours after fertilization.

up algal spores and any diatoms present (Dodd, 1957). Settled larvae were moved to fresh algae with clean water every 7 to 10 days. Water was changed every other day by carefully pipetting or siphoning off old water and adding new. By this process, one larva was raised to metamorphosis (Fig. 18) but died at 46 days owing to a cracked shell, sustained when an attempt was made to clean some algal growth from it. Three others were also raised to metamorphosis. They had lost the velum but no peristome had begun to develop.

OBSERVATIONS

Reproduction

The release of gametes in *A. testudinalis* at Noank begins in late May and early June and continues until mid- or late-July, depending upon water temperature. In 1962, water temperature at Noank was about 17.5° C. in May, and about 21.0° C. in July. Willcox (1905) found limpets spawning as late as the first week in September at Eastport, Maine, and in August at Boston. She felt that spawning ended before water reached its maximum temperature.

Sexes cannot be determined externally from about September to November. Though the normal spawning period for this limpet is during the summer, three pairs spawned in the laboratory January 28, 1963, at about 17.0° C., and normal larvae developed. However, these particular limpets had been collected in December, 1962, and the warmer temperature of the aquatic room may have stimulated gonad activity. Thus, it may be possible to obtain ripe gametes before the normal breeding season. Female gonads appear to mature sooner than male; of 49 limpets collected February 19, 1963, at Watch Hill, 10 were female and 39 were indeterminant. It appears, therefore, that *A. testudinalis* spawns once a year during the summer months, with spawning triggered by warmer temperatures.

In the Noank laboratory, different paired individuals (one male and one female per bowl) spawned at about 19.7° C. in mid- and late-June and at about 21.5° C. in July. Spawning did not occur below 17.0° C. or above 22.5° C., except in one female that spawned at 24.0° C. on July 3, 1963.

On the average, males tended to release gametes at a slightly lower temperature than females. As July approached, with water temperatures of 20.0 to 22.0° C., the time gap between male and female spawning was narrowed. In July, with temperatures of about 22.0° C., gametes were released at almost the same time and temperature.

Development of eggs and larvae

The unfertilized egg (Fig. 1) soon lost its gelatinous sheath. Only after this sheath disappeared and the eggs became spherical and opaque could fertilization occur. No fertilization membrane was observed in living or stained zygotes. Two

FIGURE 14. Lateral view of creeping larva; note absence of operculum; 161½ hours after fertilization.

FIGURE 15. Lateral view of larva withdrawn into shell; 186½ hours after fertilization.

FIGURE 16. Lateral view of veliger partially retracted; 219½ hours after fertilization.

FIGURE 17. Dorsal view of retracted veliger; note size of eyes and tentacles; 235 hours after fertilization.

FIGURE 18. Dorsal view of 42-day-old larva.

polar bodies were visible in stained material 20 minutes after fertilization and in the two- and four-celled stages. Cleavage is spiral, complete, and unequal after four cells. Two cells formed one hour after fertilization, four cells at one hour 30 minutes, eight cells with micromeres (Fig. 3) at two hours 30 minutes and 12 cells at three hours. By four hours and 40 minutes after fertilization, the micromeres had become noticeably smaller (Fig. 4).

Short tufts of cilia were evident on certain cells at 9½ hours (Fig. 5). At 13 hours, with development of an apical tuft and prototrochal cilia, the trochophore began to swim. At 25 hours (Fig. 6) a ventral circular group of cells denoting the stomodaeum, a posterior tuft of cilia forming the telotroch, and two cilia-tufted apical cells flanking the apical tuft could be seen. Neither apical tuft nor apical cells perform a locomotor function but appear to have some sensory role. Further growth of prototrochal cilia enabled the phototactic larvae to swim more actively. Changes to the veliger stage were seen at 30 hours (Fig. 7), with proliferation of the shell gland on the dorsal side and ventral flexion of the telotroch toward the foot rudiment.

The shell, with its granulated surface, grew rapidly, encapsulating the larvae by 32½ hours. At that stage, the shorter posterior cilia of the prototroch (now velum) were no longer visible. With the aid of the circular velum (Fig. 8) the larvae rotated through the water with sudden short bursts of speed. At 47 hours the first retractor muscle was visible through the shell (Fig. 9). By 52 hours the telotroch had disappeared. The successive positions of the telotroch during ventral flexion may be followed in Figures 7, 8 and 9. Though less phototactic, the veliger still swam actively and began to feed.

Torsion occurred at 61 hours. At 62½ hours the apical tuft had disappeared and an operculum was evident (Fig. 10). It may be noted in Figure 9 that the retractor muscle and foot were on opposite sides of the shell and that after torsion (Fig. 10) they were on the same side. Partial retraction of the larva into the larval shell was possible. With formation of a second retractor muscle, seen at 65 hours (Fig. 11), complete retraction could occur (Figs. 12 and 15). The foot had developed into a creeping organ, and at 85 hours after fertilization larvae were both crawling and swimming. By that time, eyespots had formed and tentacle rudiments were evident (Fig. 12). During the course of development the eyespots increased in size and the tentacles became longer and tipped with short cilia (Fig. 17). At 100 hours the intestine was well formed and the larvae swam less and crept about more. The foot was provided with numerous tiny cilia which are not shown in the figures. In crawling, the larvae appeared to have difficulty balancing the larval shell.

Little outward change occurred in stages shown in Figures 14 through 17. But at 17 days the very beginning of the peristome was present, the velum and operculum had disappeared, and the head began to look quite "limpet-like." Figure 18 shows a beginning peristome which eventually encircles the base of the larval shell. Tentacles and eyes were most like the adult and a rudimentary radula was present.

A. testudinalis larvae are planktonic for approximately four days, about 19 hours of which are spent as trochophores and nearly 56 hours as veligers. Metamorphosis appears to begin at about 15 days. Complete development from egg to newly metamorphosed adult probably occurs in six weeks.

Discussion

Comparison of reproduction and larval development among seven species of prosobranchs

Reproduction and development in *Acmaea testudinalis* are contrasted here with four other diotocardians (*Haliotis tuberculata, Patella vulgata, Patina pellucida* and *Acmaea virginea*) and two monotocardians (*Crepidula fornicata* and *Littorina littorea*). In general, all of the diotocardians listed have external fertilization, planktonic eggs, free-swimming trochophores, veliger larvae and a brief pelagic life (Lebour, 1937; Fretter and Graham, 1962). The two monotocardians have internal fertilization, egg capsules (planktonic in *L. littorea*, attached in *C. fornicata*), free-swimming veligers and an extended pelagic life (Conklin, 1897; Lebour, 1937; Thorson, 1946; Fretter and Graham, 1962). All the diotocardians and monotocardians listed are unisexual except *Patella vulgata* and *Crepidula fornicata,* which are protandric hermaphrodites (Fretter and Graham, 1962). All spawn some time during the summer, except *P. vulgata* which is a winter breeder (Crofts, 1937; Lebour, 1937; Fretter and Graham, 1962).

Of these diotocardians, *A. virginea* has the smallest egg (120 μ diameter without the gelatinous sheath) (Fretter and Graham, 1962) and *Patina pellucida* the largest (320 μ even without the sheath) (Lebour, 1937). All eggs are spawned singly in this group (Lebour, 1937; Fretter and Graham, 1962) except those of *A. testudinalis* which are laid in a mucous sheet formed by agglutination of the gelatinous sheaths about each egg.

The trochophore stage of the diotocardians is planktonic but in the monotocardians this stage develops within the egg (Fretter and Graham, 1962). All diotocardian trochophores here have a long apical tuft except *Haliotis*, which has none (Crofts, 1937), and all have a monotrochal prototroch except *Patella* in which there are two rows of cells (Fretter and Graham, 1962). No telotroch is present in *Haliotis* (Crofts, 1937). One tuft of cilia forms this structure in *Patella,* probably *Patina* (Lebour, 1937; Fretter and Graham, 1962), and *A. testudinalis;* three tufts compose the telotroch of *A. virginea* (Boutan, 1899). All have short cilia covering the apical end except *Haliotis* (Fetter and Graham, 1962) and all are top-shaped except the trochophore of *Patina* which is pear-shaped (Lebour, 1937). Of the first four listed, *Patina* has the longest trochophore (200 μ) (Lebour, 1937) and *Haliotis* the shortest (130 μ) (Crofts, 1937).

Diotocardian veligers develop a small circular velum, while that of the monotocardians becomes large and bilobed (Fretter and Graham, 1962). The larval shell in diotocardians is capsule-shaped with no coiling, except possibly in *Patella* (Fretter and Graham, 1962) and in *Haliotis* where it is initially a cap but later acquires a one and one-half whorl spiral (Lebour, 1937). Both monotocardian larval shells are definitely spiral (Fretter and Graham, 1962). From the literature (Boutan, 1899; Dodd, 1957), veligers of *P. vulgata, A. virginea* and *A. testudi-* in *Haliotis, Patella* and *Patina* (Raven, 1958), but is a quick process in *A. virginea* (two to three minutes) (Boutan, 1899) and *A. testudinalis* (less than one hour). With *Littorina* and *Crepidula,* torsion is a gradual process occurring within the egg (Fretter and Graham, 1962).

SUMMARY

1. At Noank, Connecticut, *Acmaea testudinalis* spawns from May to July.

2. Males tend to release gametes before females in June and early July, and thus in nature probably act as a stimulus to the females. However, in mid-July, males and females spawned at about the same time and temperature in the laboratory.

3. When laid, eggs of *A. testudinalis* appear to be in a thin mucous sheet formed by agglutination of the gelatinous sheath about each egg. This sheath swells on contact with water and soon disappears, leaving the egg free and ready for fertilization.

4. The photopositive trochophore larvae form 10 to 13 hours after fertilization and remain in this stage for about 19 hours.

5. Veligers develop 31 to 36 hours after fertilization and remain in that stage for about 56 hours. They become increasingly photonegative.

6. Complete development from egg to newly formed adult takes about six weeks.

LITERATURE CITED

Boutan, L., 1898. Sur le développment de l'*Acmaea virginea*. *C. R. Acad. Sci. Paris*, **126**: 1887–1889.

Boutan, L., 1899. La cause principale de l'asymetrie des mollusques gasteropodes. *Arch. Zool. Exp. et Gén., sér. 3*, **7**: 203–341.

Conklin, E. G., 1897. The embryology of *Crepidula*. *J. Morph.*, **13**: 1–226.

Crofts, D. R., 1937. The development of *Haliotis tuberculata*, with special reference to organogenesis during torsion. *Phil. Trans. Roy. Soc. London, Ser. B*, **228**: 219–268.

Dodd, J. M., 1951. Problems associated with artificial fertilization and the rearing of larvae in *Patella vulgata*. (*Rep.*) *Challenger Soc.*, **3**: 11–12.

Dodd, J. M., 1957. Artificial fertilization, larval development and metamorphosis in *Patella vulgata* L. and *Patella coerulea* L. *Pubbl. Staz. Zool. Napoli*, **29**: 172–186.

Fretter, V., and A. Graham, 1962. British Prosobranch Molluscs, Their Functional Anatomy and Ecology. No. 144. The Ray Society, London.

Fritchman, H. K., II, 1961a. A study of the reproductive cycle in the California Acmaeidae (Gastropoda). Part I. *The Veliger*, **3**: 57–63.

Fritchman, H. K., II, 1961b. A study of the reproductive cycle in the California Acmaeidae (Gastropoda). Part II. *The Veliger*, **3**: 95–101.

Fritchman, H. K., II, 1961c. A study of the reproductive cycle in the California Acmaeidae (Gastropoda). Part III. *The Veliger*, **4**: 41–47.

Fritchman, H. K., II, 1962. A study of the reproductive cycle in the California Acmaeidae (Gastropoda). Part IV. *The Veliger*, **4**: 134–140.

Grant, A. R., 1937. A systematic revision of the genus *Acmaea* Eschscholtz, including consideration of ecology and speciation. Ph.D. Thesis, University of California, Berkeley.

Lebour, M. V., 1937. The eggs and larvae of the British Prosobranchs with special reference to those living in the plankton. *J. Mar. Biol. Assoc.*, **22**: 105–166.

Morse, E. S., 1910. An early stage of *Acmaea*. *Proc. Soc. Nat. Hist. Boston*, **34**: 313–323.

Orton, J. H., A. J. Southward and J. M. Dodd, 1956. Studies on the biology of limpets. Part II. The breeding of *Patella vulgata* L. in Britain. *J. Mar. Biol. Assoc.*, **35**: 149–176.

Orton, J. H., and A. J. Southward, 1961. Studies on the biology of limpets. Part IV. The breeding of *Patella depressa* Pennant on the North Cornish coast. *J. Mar. Biol. Assoc.*, **41**: 653–662.

Patten, W., 1885. Artificial fecundation in Mollusca. *Zool. Anz.*, **8**: 236–237.

Patten, W., 1886. The embryology of *Patella*. *Arb. Zool. Inst. Wien*, **6**: 149–174.

Raven, Chr. P., 1958. Morphogenesis: the Analysis of Molluscan Development. Pergamon Press, New York.

Smith, F. G. W., 1935. The development of *Patella vulgata*. *Phil. Trans., Roy. Soc. London, Ser. B*, **225**: 95–125

THOMPSON, W. F., 1912. The protoconch of *Acmaea*. *Proc. Acad. Nat. Sci.*, **64**: 540–544.

THORSON, G., 1935. Studies on egg capsules and development of Arctic marine prosobranchs. *Meddel. om Groenland*, **100**: 1–71.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Kom. Havundersøg., Kbh., ser. Plankton*, **4**: 1–523.

WILLCOX, M. A., 1898. Zur Anatomie von *Acmaea fragilis* Chemnitz. *Jen. Zeitschr. f. Naturwiss.*, **32**: 411–456.

WILLCOX, M. A., 1900a. Notes on the anatomy of *Acmaea testudinalis* Müller. *Science*, **11**: 171.

WILLCOX, M. A., 1900b. Hermaphroditism among the Docoglossa. *Science*, **12**: 230–231.

WILLCOX, M. A., 1905. Biology of *Acmaea testudinalis* Müller. *Amer. Nat.*, **39**: 325–333.

WILLCOX, M. A., 1906. Anatomy of *Acmaea testudinalis* Müller. Part I. Introductory material—external anatomy. *Amer. Nat.*, **40**: 171–187.

LIFE-HISTORY AND PHOTOPERIODIC RESPONSES OF NYMPHS OF TETRAGONEURIA CYNOSURA (SAY)[1]

PAUL E. LUTZ[2, 3] AND CHARLES E. JENNER

Department of Zoology, University of North Carolina, Chapel Hill, North Carolina

Many biological aspects of the Odonata are poorly known; for species in this country, problems involving regulation of development by seasonal changes have been neglected especially. Interest in seasonal regulation in the Odonata has been stimulated recently as a result of detailed investigations by Corbet on the dragonfly, *Anax imperator* (1956a, 1957a), and on other species (1955a; 1956b, 1956c; 1957b, 1957c). Summaries of his interesting ideas concerning seasonal regulation are to be found in two recent volumes (Corbet *et al.*, 1960; Corbet, 1963).

Life-history studies on dragonflies are neglected in spite of the many published reports on the subject. Most so-called life-history or life-cycle studies have been concerned with rearing nymphs to adulthood under artificial, and usually variable, conditions. A more desirable method of studying patterns of nymphal development is to sample regularly populations in nature throughout the year. This method has been utilized effectively by Corbet (1956b; 1957a, 1957b, 1957c) on several British species, by Eller (1963) on *Pachydiplax* in North Carolina, and in a limited way by Kormondy (1959) on *Tetragoneuria* in Michigan.

The experimental studies of Corbet (1956a), Jenner (1959), Lutz and Jenner (1960), Schaller (1960), and Montgomery and Macklin (1962) have shown that day-length is an important factor in regulating life-cycles of several species of Odonata. In preliminary experiments on *Tetragoneuria cynosura,* Jenner (1959) and Lutz and Jenner (1960) subjected over-wintering, last-stage nymphs to short and long photoperiods of 11 and 14 hours, respectively. On the longer photoperiod nymphal development was completed in one-third the time required by nymphs on the shorter photoperiod.

The present investigation was carried out to determine the pattern of development in *T. cynosura* nymphs in nature, and to study seasonal differences in photoperiodic responses. Since nothing is known about the threshold light intensity in Odonata photoperiodism or the minimal number of consecutive inductive photoperiods required for a response, attention was also given to these matters in this study.

MATERIALS AND METHODS

Field studies were carried out at University Lake, an impoundment constructed in 1931 and located three miles west of Chapel Hill, North Carolina. The area

---

[1] Supported in part by grants from the University of North Carolina Research Council and the U. S. Public Health Service (E 356).

[2] Part of a dissertation submitted to the faculty of the Department of Zoology in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Present address: Department of Biology, University of North Carolina at Greensboro.

was selected because of accessibility and the high density of nymphs of *T. cynosura*. The shore line of the study area measured approximately 43 meters and contained numerous sedges, grasses, rushes and small shrubs. The bottom consisted primarily of abundant decaying vegetation resulting from dense rooted plants (chiefly *Sagittaria* sp. and *Saururus cernus*) and bordering trees (*Platanus occidentalis*, *Alnus serrulata* and *Salix* spp.).

Twenty-one collections of nymphs were made at two- to four-week intervals from August 12, 1960, until August 16, 1961. Numbers of individuals varied from 10 to 116 per collection. Animals comprising nine collections were measured in the field and returned to their approximate habitat. Animals in the remaining collections were brought to the laboratory, allowed to come slowly to a temperature of 22° C. and then measured, using a Bogusch measuring slide and binocular microscope. Measurements were made on total body length from the most anterior part of the labium (at rest) to the posterior ends of the lateral spines of the ninth abdominal segment.

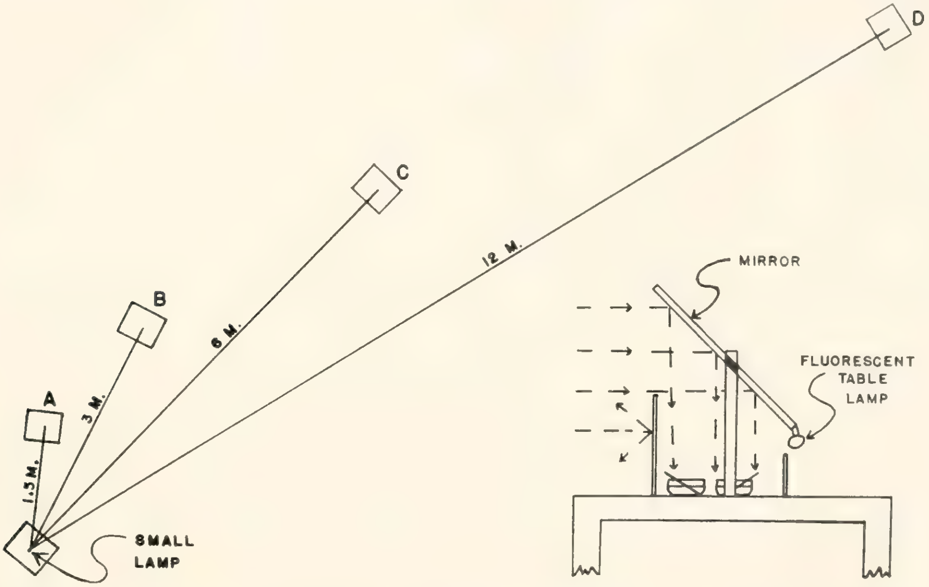


FIGURE 1. Plan of stations employed in light-intensity experiments and a diagrammatic representation of arrangements at each station.

All experimental animals were maintained in individual fingerbowls 10 cm. in diameter and were fed *ad libitum* on *Daphnia magna* with supplemental feedings of *Daphnia pulex* and tubificid worms. A 12-cm. length of dowel, 1 cm. in diameter, was added to provide a means of exit for the nymphs from the water at emergence.

All nymphs, except those utilized in light-intensity experiments, were housed in light-proof cabinets, each measuring 0.61 × 1.12 × 0.85 meters, in a controlled-temperature room at about 22° C. Photoperiods of 14 hours (long day) and 11 hours (short day) were employed. Light was supplied by two 30-watt daylight fluorescent lamps operated by automatic time switches; intensities ranged from 270 to 430 lux. When the cabinets were closed, air from the room was circulated through them by a centrifugal blower *via* a system of light-proof ducts; temperature variations within the cabinets remained within ±1° C. of room temperature.

Light-intensity experiments, utilizing the same facilities reported by Paris and Jenner (1959), were conducted according to the plan illustrated in Figure 1. A fairly constant temperature of about 22° C. was maintained, although occasional extremes up to ±8° C. were recorded. Individual station lamps (15-watt, daylight fluorescent), controlled by a single time switch set for an 11-hour photoperiod, delivered an intensity of 375 lux at bowl level. Three additional hours of low-intensity light, provided by a small lamp of 6 watts and controlled by a separate timer, increased the duration of the photoperiod to 14 hours for the animals at Stations A, B, C and D (Fig. 1). Therefore, animals at Stations A through D received an identical 11-hour photoperiod, followed by three additional hours during which time the light intensity was progressively lower from Stations A to D. Nymphs which served as long-day controls were located on the table with the small lamp where they were subjected to an intensity of about 5.5 lux for the 14-hour period. Short-day controls were placed in a light-proof box near a station lamp and were covered manually at the termination of the 11-hour photoperiod and opened in the dark.

A second intensity experiment was executed in the same manner except that filters (eight thicknesses of #1 Whatman Filter Paper, which reduced intensities by 93%) were used at the four stations. The filters, located about 8 cm. above the nymphs, were in place only during the three-hour period when the 6-watt lamp was on.

RESULTS

Seasonal cycle

To illustrate the nature of the seasonal cycle of this species, results of the sampling program are given as relative length-frequency histograms in Figure 2a and 2b. Data for different size groups are given as percentage of the entire collection so that comparisons can be made more easily between samples of different sizes. The final four instars were identified readily on the basis of length, but earlier instars could not be separated by this method. Correct recognition of later instars was substantiated by rearing these individuals to emergence in the laboratory.

Most of the population had a one-year life-cycle, as is obvious from Figure 2. The collection of August 12, 1960, consisted of three instars with the antepenultimate being most abundant. Progressive development during August, September, and early October was demonstrated clearly and by October 22, all nymphs sampled were in the final instar. Winter collections consisted almost exclusively of last-stage nymphs.

By March 24, 1961, all last-stage nymphs showed morphological changes associated with impending emergence; these changes included swelling of the wing sheaths and the separation of the mesothoracic wing sheaths. The emergence period extended from April 5 until about May 13. Following this period a collection was made on May 18, employing approximately the same degree of effort as that used in the March 24 collection, but yielding only 10 nymphs. These nymphs obviously

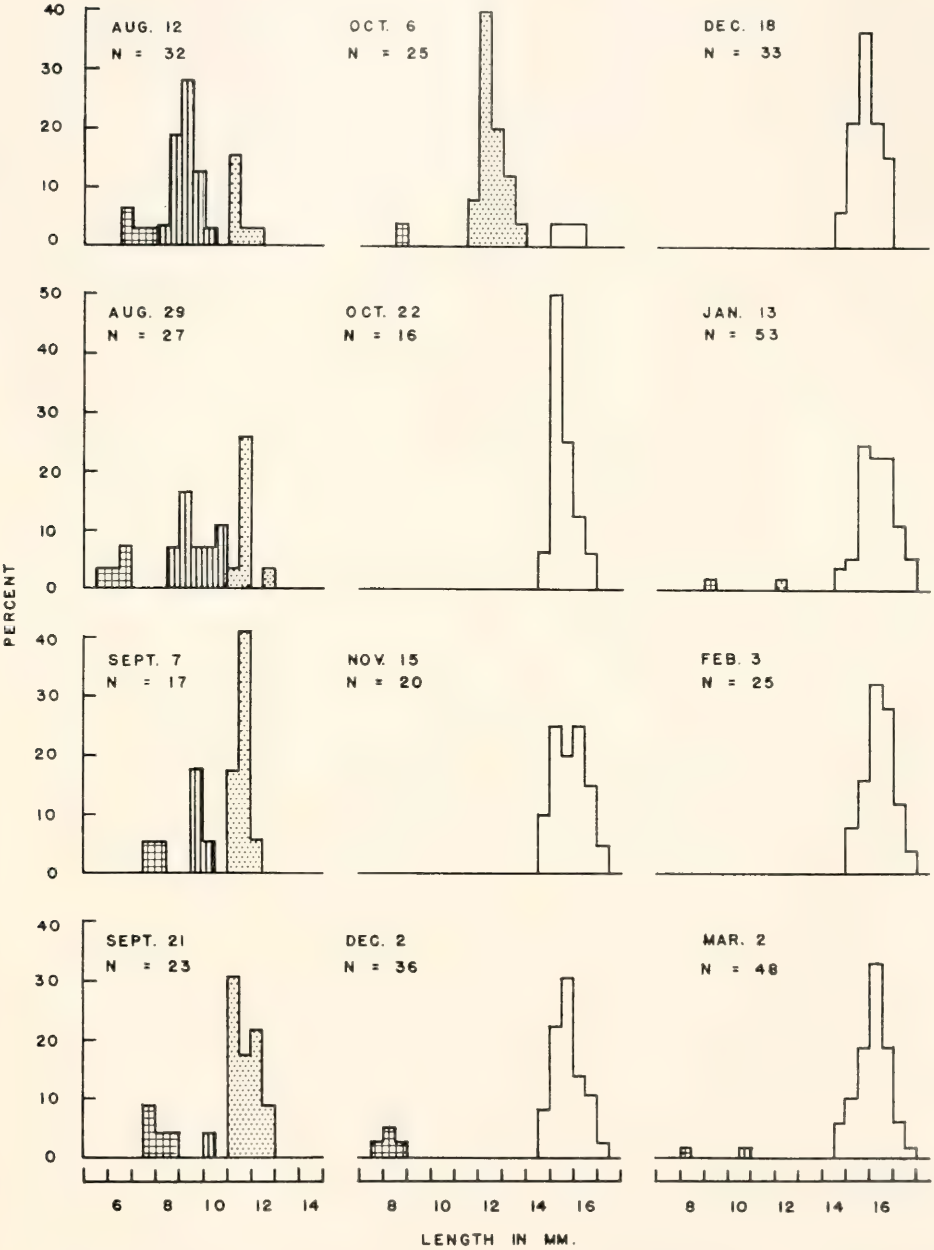


FIGURE 2A. Relative length-frequency histograms of *Tetragoneuria cynosura* nymphs collected in 1960–1961. Cross-hatched area = stage before antepenultimate instar; vertical lined area = antepenultimate instar; stippled area = penultimate instar; clear area = ultimate instar.

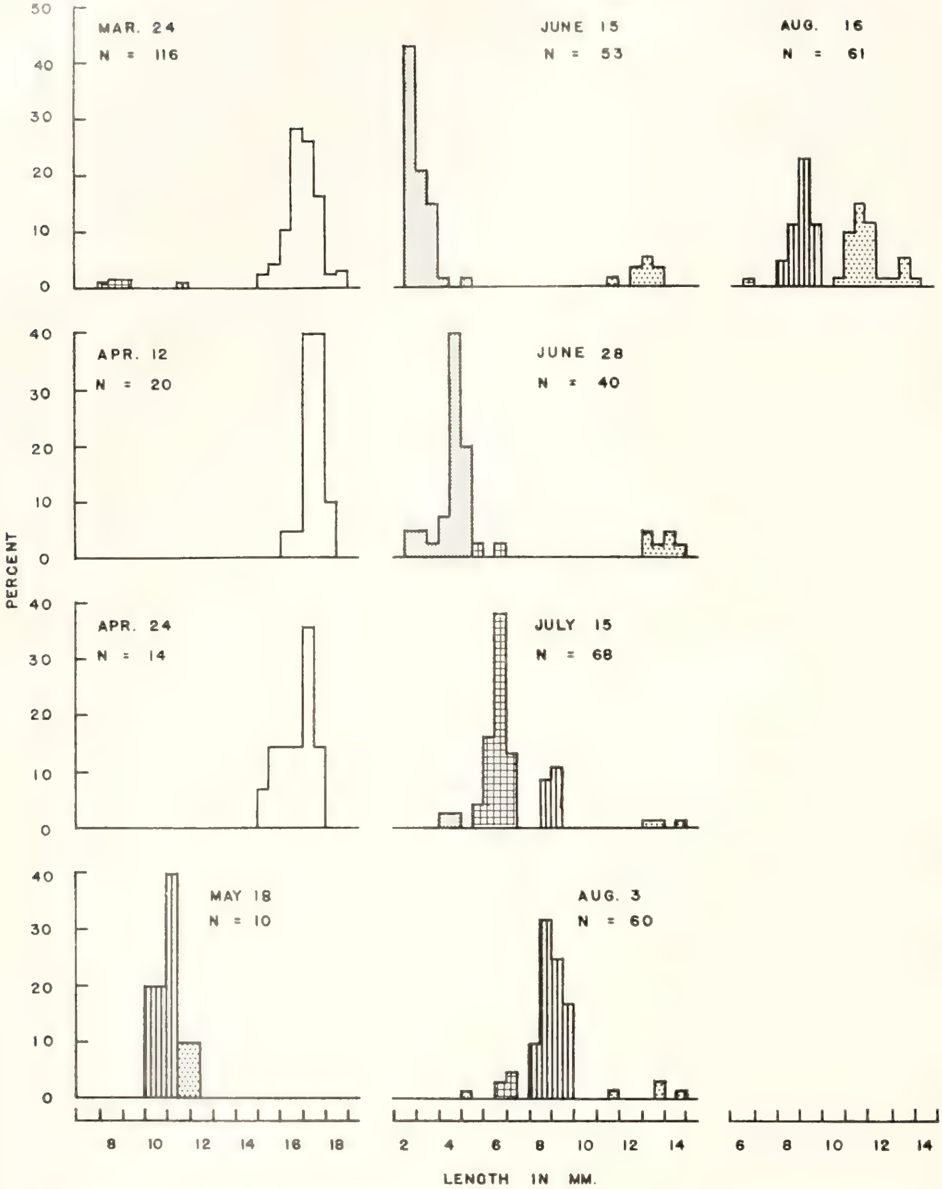


FIGURE 2B. Relative length-frequency histograms of *Tetragoneuria cynosura* nymphs collected in 1960–1961. Finely stippled area = early stages; cross-hatched area = stage before antepenultimate instar; vertical lined area = antepenultimate instar; coarsely stippled area = penultimate instar; clear area = ultimate instar.

had passed the winter in some stage short of the final instar. A small number of such individuals appeared in collections on December 2, January 13, March 2 and March 24.

The new generation was the dominant element in the June 15 collection, constituting 90% of the sample. This 0-year class developed rapidly during the summer, but remnants of the one-year class persisted as a small group in the penultimate stage. By August 16 recruitment into this latter stage had occurred from the 0-year class and a population distribution was again achieved, similar to the August collection of the previous year.

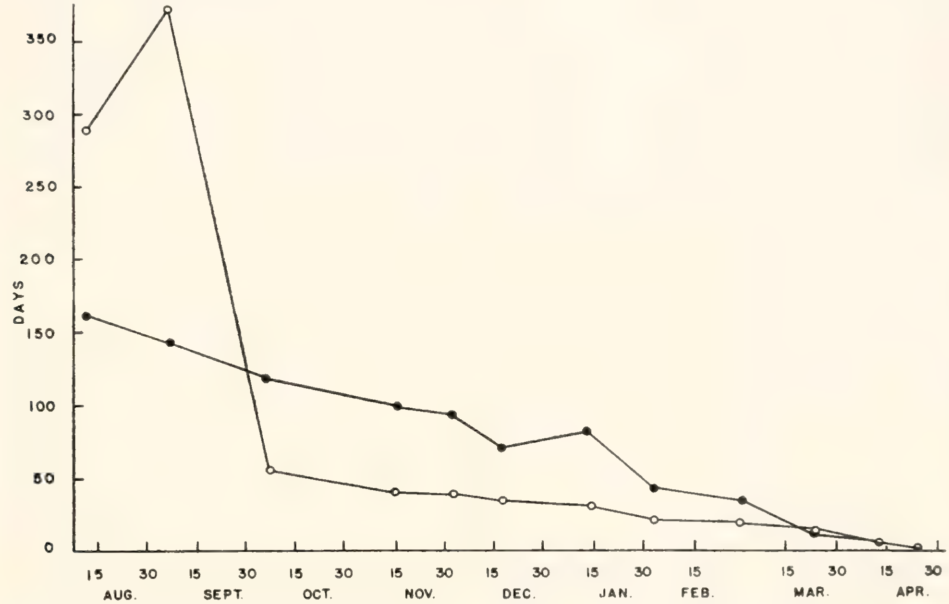


FIGURE 3. Average duration of time spent in the final instar in the laboratory by nymphs on 11- and 14-hour photoperiods. Open circles = 14-hour; closed circles = 11-hour.

Most individuals took only one year to complete their life-cycle, but a small part of the population required two years to undergo nymphal development and were, therefore, semivoltine. The percentage of retarded individuals in the samples varied from 0% to 15.2% and probably constituted 5–10% of the population.

Seasonal changes in response to photoperiod

In preliminary experiments Lutz and Jenner (1960) noted seasonal differences in response by nymphs to photoperiods of 11 and 14 hours. The response measured was time spent in the laboratory from collection to emergence. These observations suggested the desirability of initiating experiments throughout a more extensive period. Twelve experiments were started between August 12, 1960, and April 24, 1961, using the two contrasting photoperiods. All nymphs in the collections of August 12 and September 7, and all but two individuals in the October 6 collection, spent the entire final instar in the laboratory. Data for nymphs from the remaining collections pertain to time spent in the laboratory from collection to emergence since all were already in the final instar when collected. Results of these experiments are shown in Figure 3.

An abrupt change in response was shown for nymphs placed on long-day conditions. Nymphs collected on August 12 and September 7 and kept on the 14-hour

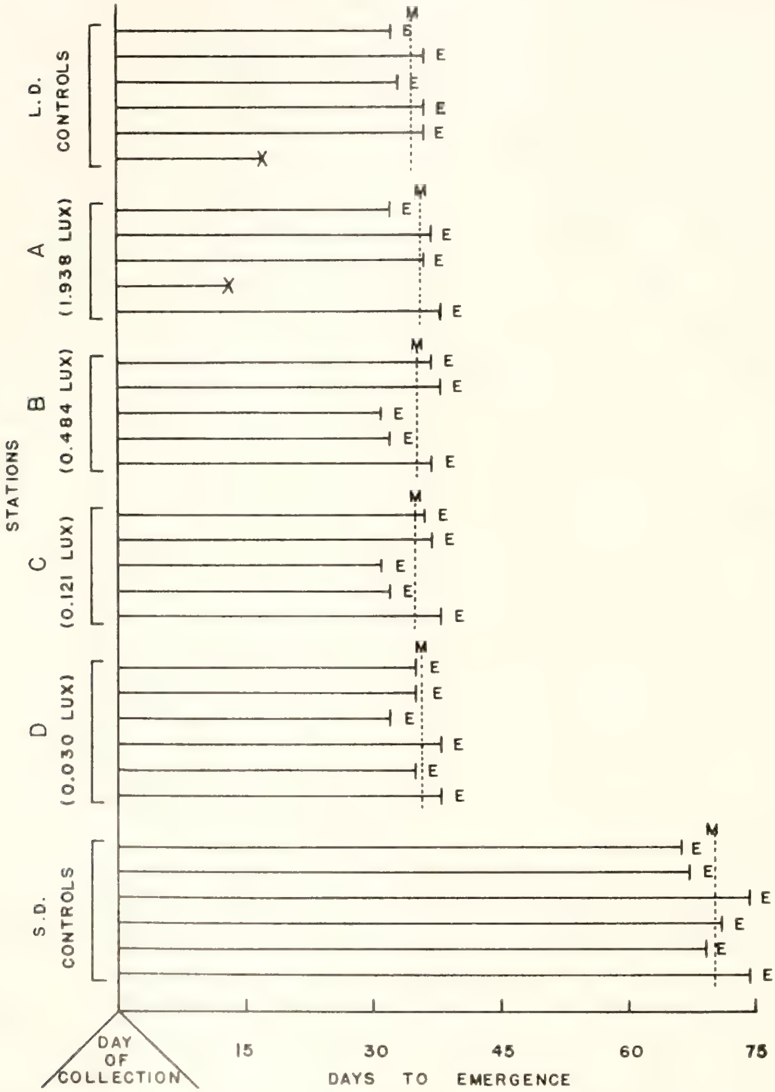


FIGURE 4. Days spent from collection to emergence by individual nymphs maintained on a basic 11-hour photoperiod plus three additional hours of low light intensity. M = mean; horizontal line ending with short vertical line followed by "E" = emerged here.

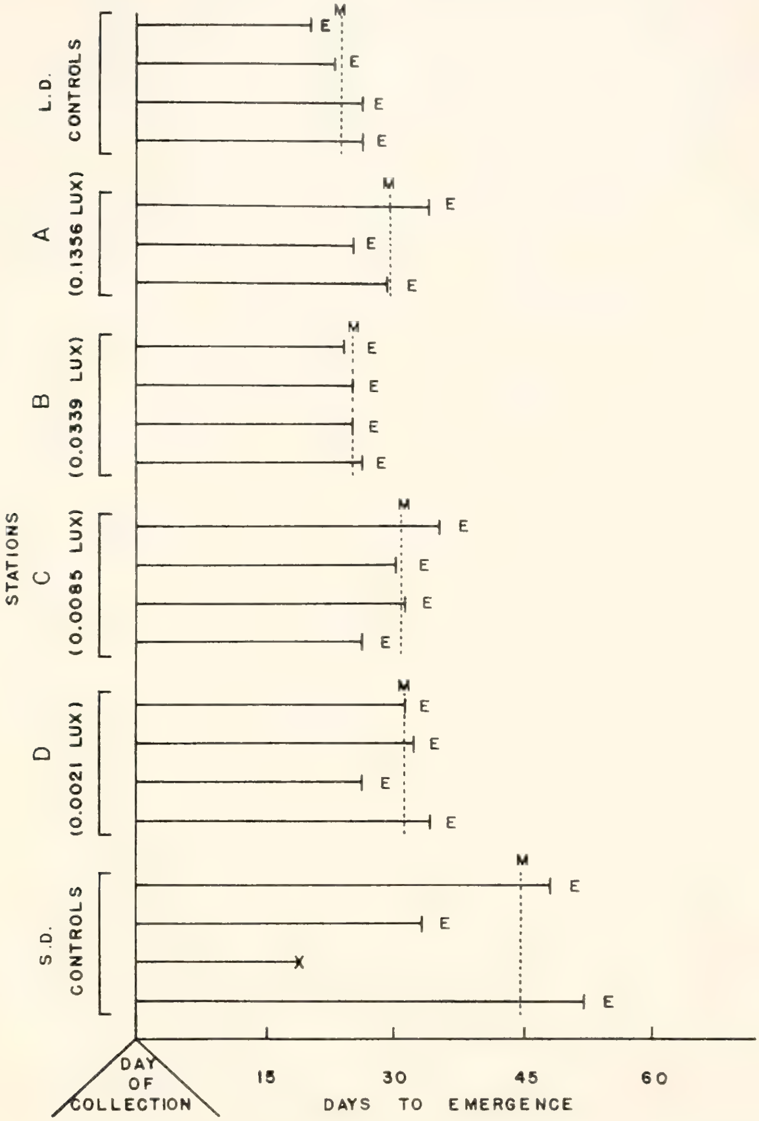


FIGURE 5. Days spent from collection to emergence by individual nymphs maintained on a basic 11-hour photoperiod plus three additional hours of very low light intensity. M = mean; horizontal line ending with short vertical line followed by "E" = emerged here.

day required approximately twice the time to pass through the final instar as did the short-day animals collected at the same time. In subsequent collections, however, the response of long-day individuals was much faster than that of nymphs on an 11-hour photoperiod. This dramatic reversal in response occurred during the period of the autumnal equinox.

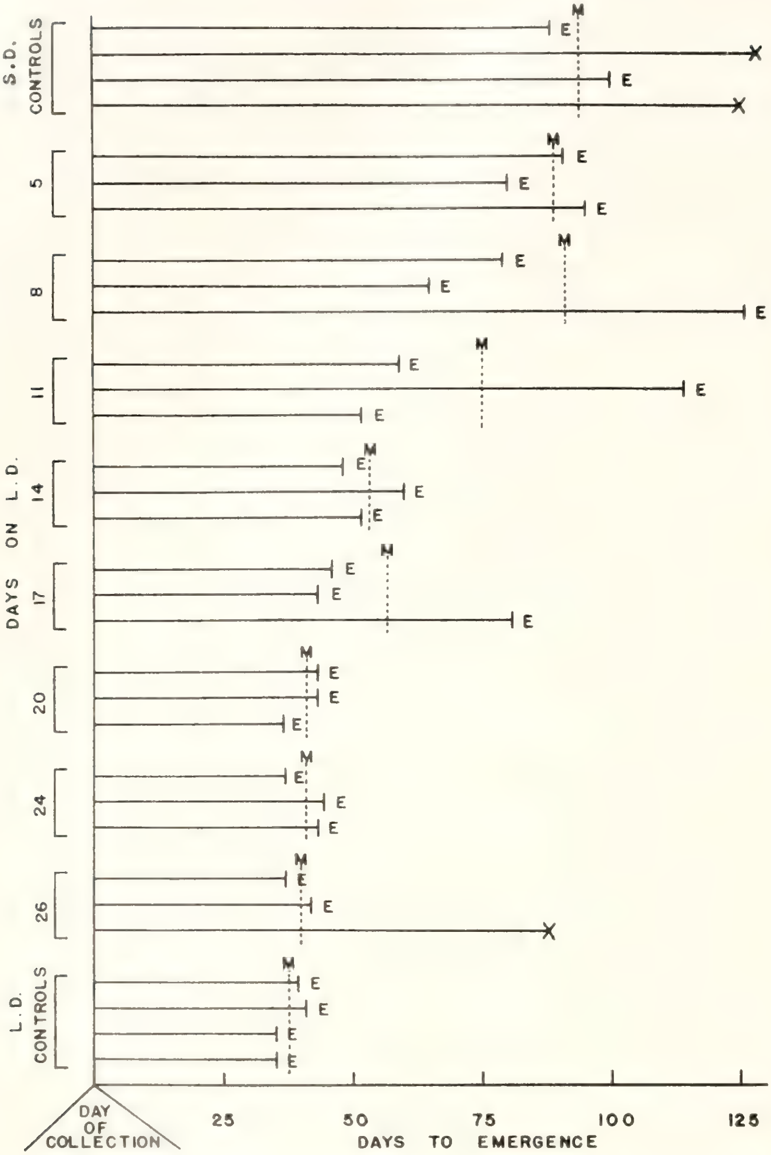


FIGURE 6. Individual responses of nymphs to an 11-hour day-length after being subjected to a varying number of long-day cycles. M = mean; horizontal line ending with short vertical line followed by "E" = emerged here.

The time required for short-day nymphs to emerge was progressively less throughout the entire experimental period. Following the autumnal equinox, differences in response to the two photoperiods decreased slowly until they were negligible for the last three collections.

Light intensity experiments

Two experiments were conducted in an attempt to determine the threshold light intensity for a long-day response. In the first experiment nymphs collected on December 18, 1960, were subjected to low experimental intensities ranging from approximately 1.94 to 0.03 lux. Days from collection to emergence for experimental and control animals are given in Figure 4. The results clearly show all experimental intensities to be above the threshold for a long-day response. A second experiment, therefore, was conducted, employing lower intensities that ranged from approximately 0.136 to 0.002 lux. Animals collected on February 3, 1961, were employed in the experiment; the results are given in Figure 5. Average days to emergence for nymphs subjected to these experimental intensities were closer to the mean for long-day controls than to that for the short-day animals. These results, although more variable than in the earlier experiment, indicate that the threshold intensity for a photoperiodic response in this species is exceedingly low, perhaps below 0.002 lux.

Inductive cycle experiment

Using final-stage nymphs collected on December 2, 1960, an experiment was conducted to determine the minimal number of 14-hour cycles (*i.e.,* 14 hours of light and 10 of darkness in each 24 hours) necessary for a long-day response. The animals were placed on a 14-hour photoperiod and, at intervals, three nymphs were transferred to an 11-hour photoperiod and maintained there until emergence. The results of this experiment are shown in Figure 6. The mean time to emerge for those nymphs subjected to five and eight long-day cycles was similar to the mean of the 11-hour controls. Animals experiencing 20 or more days on the 14-hour photoperiod responded similarly to the long-day controls. Nymphs subjected to 11, 14 and 17 long-day cycles exhibited a response intermediate between the two controls. Despite the small number of animals used and variations in response, the results suggest that more than 8, but less than 20, long-day cycles were necessary to produce a long-day response.

Discussion

Corbet (1963) has indicated that a diapause stage is a major factor in synchronizing nymphal development and emergence. Corbet *et al.* (1960) have classified British Odonata on the basis of position of the diapause stage, pattern, and duration of emergence periods, and mechanisms by which development and emergence are synchronized. They have designated as "spring species" those which emerge early in the season and have a diapause stage in the final instar. Emergence is characterized by an early peak due to the synchronizing effect of a final instar diapause. "Summer species" emerge later and a diapause stage (if there is one) does not occur in the ultimate instar. The emergence period in these forms is more widely dispersed temporally, due to a lesser degree of synchronization caused by the lack of a final instar diapause. Corbet (1957c) has postulated a theory of lower temperature thresholds which he believes would account for the degree of synchronization that is exhibited by "summer species."

Life-cycles of only a few dragonflies have been determined by regular sampling of natural populations. The present investigation is apparently the first published report for a species in this country. Corbet (1956b; 1957a, 1957b, 1957c) has studied life-cycles of several British species by this method, including a detailed study of *Anax imperator* (Corbet, 1957a). He observed a two-year life-cycle in *Anax,* although a small percentage developed precociously and was, therefore, univoltine. Employing a large sampling program, Eller (1963) has made an excellent study of *Pachydiplax longipennis* and found this "summer species" has a life-cycle of one year.

For *Tetragoneuria cynosura* the only information concerning the pattern of nymphal development in nature was given by Kormondy (1959), who reported a two-year life-cycle for this species in Michigan. His evidence was not satisfactory, however, since it was based on inadequate sampling and "extrapolation" from laboratory rearing. Our study conducted in North Carolina has shown conclusively that development for most nymphs of *T. cynosura* requires one year. The final instar was achieved generally by October and served as an over-wintering diapause stage. A well-synchronized emergence period occurred in the spring, and the position of diapause and emergence patterns characterize *T. cynosura* as a "spring species" in Corbet's classification. A small percentage of nymphs exhibited retarded growth and required two years for development. A pattern of development similar to that found in *T. cynosura* in this study has not been reported previously for a species of this insect order.

Corbet (1955b; 1956a) first reported a role of day-length in inducing diapause in Odonata. He concluded that the sign of change in successive day-lengths, *i.e.,* whether they are increasing or decreasing, influences the seasonal incidence of diapause in *Anax.* This conclusion should not be accepted without question since the results of his experiments, described as preliminary, were quite variable. Further, an assumption was made, as yet untested, that photoperiod was without influence once diapause was entered. Jenner (1959) and Lutz and Jenner (1960) demonstrated conclusively that an absolute photoperiod of 14 hours promoted development in diapausing, final-instar nymphs of *T. cynosura,* whereas an 11-hour day delayed development. Similar results have been reported by Montgomery and Macklin (1962) on *Neotetrum pulchellum.* The experiments by Schaller (1960) on *Aeschna cyanea* did not permit the separate effects of photoperiod and temperature to be determined.

Perhaps the most interesting aspect of this study concerns the observed differences in seasonal response to the two photoperiods. Experiments started in August and September showed that the duration of the final instar in nymphs maintained on long days was about twice that of short-day nymphs. A similar delay under long-day conditions must also occur in nature in the penultimate instar, which would account for the developmental arrest in the older nymphs (one-year class) occurring in summer collections. Day-length conditions during the period of the autumnal equinox must serve as a synchronizing agent and promote entry into the final instar by nymphs of both year classes. Following this period, short photoperiods must now induce delayed development in ultimate instar nymphs, thus insuring that they will enter the winter period in an appropriate stage of developmental arrest.

Delayed development was evident in long-day nymphs from the August 12 and September 7 collections and also by penultimate nymphs in nature during the summer. This developmental arrest may represent a phenomenon comparable to that known for certain birds which have a summer refractory period to photoperiodic induction. The role of day-length in terminating this refractory condition in *Tetragoneuria cynosura* has not, as yet, been clarified. An exploratory experiment, not reported in detail here, was conducted in which 5 nymphs of the collection of August 12, 1960, were subjected to alternating 15-day intervals of long and short photoperiods. These nymphs completed their development in less time than corresponding nymphs on either the long or short photoperiods. However, further experiments are required to clarify the critical factor or factors in operation.

The present study has demonstrated that nymphs respond to extremely low light intensities, probably below 0.002 lux. This value is lower than those reported by De Wilde (1962) in various insects and by Paris and Jenner (1959) in the midge, *Metriocnemius*. The results of the inductive cycle experiment indicate that the effect of a long day is not irreversible initially, but requires at least eight long-day cycles to produce a long-day response.

SUMMARY

1. Most nymphs of *Tetragoneuria cynosura* required one year for the completion of nymphal development in nature. Rapid growth occurred in the summer months and by the end of October, the final instar was attained which served as an overwintering diapause stage; emergence occurred in early April and May. A small percentage of the population (5–10%) exhibited growth retardation and had a life-cycle of two years. These individuals spent the first winter in stages short of the last, the summer in the penultimate stage, and the second winter in the last stage.

2. Striking differences occurred in seasonal response to photoperiods of 11 and 14 hours. Durations of the final instar for nymphs collected in August and September were much greater on the longer day-length. Following the fall equinox period, a reversal in response occurred, with the longer photoperiod inducing more rapid development in fall and winter collections. Differences in rates of response became progressively less as time of emergence approached. These results were utilized in an attempt to explain the role of photoperiod in controlling seasonal nymphal development in this species.

3. The threshold light intensity necessary to elicit a photoperiodic response was found to be extremely low, probably below 0.002 lux. More than eight, but less than twenty, long-day cycles were necessary to induce irreversibly a response similar to that of the long-day controls.

LITERATURE CITED

Corbet, Philip S., 1955a. The larval stages of *Coenagrion mercuriale* (Charp.) (Odonata: Coenagriidae). *Proc. Roy. Entomol. Soc. London, Ser. A,* **30**: 115–126.

Corbet, Philip S., 1955b. A critical response to changing length of day in an insect. *Nature,* **175**: 338–339.

Corbet, Philip S., 1956a. Environmental factors influencing the induction and termination of diapause in the emperor dragonfly, *Anax imperator* Leach (Odonata: Aeshnidae). *J. Exp. Biol.,* **33**: 1–14.

CORBET, PHILIP S., 1956b. The life-histories of *Lestes sponsa* (Hansemann) and *Sympetrum striolatum* (Charpentier) (Odonata). *Tijdschr. Entomol.*, **99**: 217–229.

CORBET, PHILIP S., 1956c. The influence of temperature on diapause development in the dragonfly *Lestes sponsa* (Hansemann) (Odonata: Lestidae). *Proc. Roy. Entomol. Soc. London, Ser. A*, **31**: 45–48.

CORBET, PHILIP S., 1957a. The life-history of the emperor dragonfly *Anax imperator* (Leach) (Odonata: Aeshnidae). *J. Anim. Ecol.*, **26**: 1–69.

CORBET, PHILIP S., 1957b. The life-histories of two spring species of dragonfly (Odonata: Zygoptera). *Entomol. Gaz.*, **8**: 79–85.

CORBET, PHILIP S., 1957c. The life-histories of two summer species of dragonfly (Odonata: Coenagriidae). *Proc. Zool. Soc. London*, **128**: 403–418.

CORBET, PHILIP S., 1963. A Biology of Dragonflies. Quadrangle Books, Inc., Chicago. 247 pp.

CORBET, PHILIP S., CYNTHIA LONGFIELD AND N. W. MOORE, 1960. Dragonflies. Collins, London, 260 pp.

DE WILDE, J., 1962. Photoperiodism in insects and mites. Pp. 1–26 in: Steinhaus and Smith (Ed.), Annual Review of Entomology, Annual Reviews, Inc., Palo Alto, Calif.

ELLER, JAMES GERALD, 1963. Seasonal regulation in *Pachydiplax longipennis* (Burmeister) (Odonata: Libellulinae). Unpublished Ph.D. dissertation, University of North Carolina.

JENNER, CHARLES E., 1959. The effect of photoperiod on the duration of nymphal development in several species of Odonata. *Bull. Assoc. Southeastern Biol.*, **6**: 26.

KORMONDY, EDWARD J., 1959. The systematics of *Tetragoneuria*, based on ecological, life history, and morphological evidence (Odonata: Corduliidae). *Misc. Publ. Mus. Zool., Univ. of Michigan, No. 107*, 79 pp.

LUTZ, PAUL E., AND C. E. JENNER, 1960. Relationship between oxygen consumption and photoperiodic induction of the termination of diapause in nymphs of the dragonfly *Tetragoneuria cynosura*. *J. Elisha Mitchell Sci. Soc.*, **76**: 192–193.

MONTGOMERY, B. ELWOOD, AND JERRY M. MACKLIN, 1962. Rates of development in the later instars of *Neotetrum pulchellum* (Drury) (Odonata, Libellulidae). *Proc. North Cent. Branch, Entomol. Soc. Amer.*, **17**: 21–23.

PARIS, OSCAR H., AND CHARLES E. JENNER, 1959. Photoperiodic control of diapause in the pitcher-plant midge, *Metriocnemius knabi*. Pp. 601–624 in: Photoperiodism and Related Phenomena in Plants and Animals. *Amer. Assoc. Adv. Sci.*, Washington, D. C.

SCHALLER, F., 1960. Étude du développement post-embryonnaire d'*Aeschna cyanea* Mull. *Ann. Sci. Nat., 12e Ser.*, **2**: 753–868.

SUCCINOXIDASE ACTIVITY IN HOMOGENATES OF DUGESIA DOROTOCEPHALA

WILLIAM L. MENGEBIER AND MARIE M. JENKINS

Department of Biology, Madison College, Harrisonburg, Virginia

Studies pertaining to the respiratory activity of invertebrates have been quite extensive, as demonstrated by the work of Ludwig and Barsa (1956) and Akov and Guggenheim (1963) on insects, Nielsen (1961) on enchytraeids and nematodes, and Ghiretti-Magaldi, Giuditta and Ghiretti (1958) on cephalopods. At the cellular level, the recent publication by Bliss and Skinner (1963) contains the data presently available as to both endogenous and respiratory enzyme activities of invertebrates. Absent, however, are any data dealing with respiratory activity at the cellular level in Platyhelminthes, specifically the free-living flatworms.

That these animals have an active oxygen consumption is well documented (Allen, 1919; Hyman, 1919; Bolen, 1937; and Jenkins, 1960). Furthermore, it seems to be accepted that the normal pathways of aerobic respiration—dehydrogenases, cytochromes, and oxidases—are present and active (Hyman, 1951). Insofar as can be determined, however, this acceptance of aerobiosis in planarians is based on indirect evidence. A significant decrease in oxygen consumption by whole animals after the addition of KCN, which is generally considered to be specific for cytochrome oxidase, has been reported by Allen (1919) and Bolen (1937). Hammen and Lum (1962) and Smith and Hammen (1963) have concluded, through the use of p-chloromercuribenzoic acid, that malate, a normal constituent of the Krebs cycle, is present in the cells of planarians. Finally, the high oxygen consumption of planarians during carbohydrate metabolism is thought by von Brand (1936) to be indicative of aerobic respiration. Direct assays of respiratory enzyme activity, however, have not been reported.

The purpose of this study was to assay homogenates of whole specimens of *Dugesia dorotocephala* for succinoxidase activity, utilizing the Warburg technique. Since the succinoxidase system is generally accepted as a valid criterion for the presence of the Krebs cycle, it was felt that positive evidence of such activity would serve to corroborate the indirect evidence now utilized to prove the existence of normal channels of aerobic respiration in these flatworms.

METHODS AND MATERIALS

Specimens of an asexual race of *Dugesia dorotocephala* were collected from Massanetta Springs, Virginia, and kept in aerated tanks at a temperature of 18.5–19° C.

With the exception of the first series of experiments, in which all animals were starved for 48 hours prior to an experiment, regular feedings of raw liver were so scheduled that all worms had food available until two hours before homogenization. This latter feeding procedure has been reported by Bolen (1937) as initiating maximum oxygen consumption by whole worms.

Twenty-five to 30 planarians were blotted dry with filter paper and weighed, then a 10% distilled water homogenate was prepared, using a glass tube with a Teflon pestle. The succinoxidase activity of these homogenates was determined according to the method of Schneider and Potter (1943); all experiments were carried out at 25° C. Manometer readings were taken for three 15-minute intervals, after an original equilibration period of 10 minutes. The most reproducible results were gained from the second 15-minute interval. Dry weights for Q_{O_2} determinations (μl. O_2/mg. dry wt./hr.) were obtained by placing aliquots of all homogenates in an oven overnight at 103° C.

In the experiments dealing with pH values and succinoxidase activity, phosphate buffers with pH values of 5.0, 6.0, 7.0, 7.4, 7.6, 7.9, 8.3, 8.7, 10.4 and 11.4 were prepared. All pH measurements were made with a Beckman Model 72 pH meter. Respiratory inhibitors used were 2×10^{-3} KCN and 5×10^{-3} urethane.

RESULTS AND DISCUSSION

Succinoxidase activity in homogenates of *Dugesia dorotocephala* was found to be present, with Q_{O_2} values for animals starved for 48 hours prior to experimentation averaging 2.87, and for fed animals 8.58.

These original experiments were all run at a pH of 7.4, the optimum reported for the succinoxidase system in the Handbook of Biological Data (1956). Planarians, however, have the ability rapidly to convert their external medium to a constant pH (Hyman, 1951), which in our laboratory was found to be 7.9–8.0. To determine whether the external pH in any way affects the internal environment insofar as optimum enzyme conditions are concerned, a series of assays was run utilizing the varying pH values given earlier in this paper. A minimum of nine assays was made for each pH value plotted beyond these limits. Mean Q_{O_2} values plotted against pH are shown in Figure 1. As can be seen, the usual bell-shaped curve normally found when plotting enzyme activity against pH was realized. The peak of the curve, however, shows that maximum activity occurred at a pH of 8.3 rather than at 7.4, the figure normally accepted as being optimum.

This high pH value for optimum succinoxidase activity in *Dugesia dorotocephala* is worthy of note. It is generally agreed that the sensitivity of enzymes to changes in pH is due to their protein nature, and that, usually, optimum enzyme activity takes place within the narrow limits of a given hydrogen ion concentration. It must be pointed out, however, that the evidence for this is based primarily on work done with vertebrate tissue or isolated enzymes. Invertebrates have not received the same attention, and since the majority of these species are found in environments in which physical factors such as pH and temperature may diverge widely from a given value, it seems plausible to assume that criteria other than those applied to vertebrates may be considered. Steinbach's studies on ionic and water content of planarians (1962) drew attention to the fact that little is known about the mechanisms whereby these organisms can adjust and maintain water and ionic balance in such varying habitats. If rather wide external fluctuations in the environment of planarians are the rule, it would seem likely that greater latitude

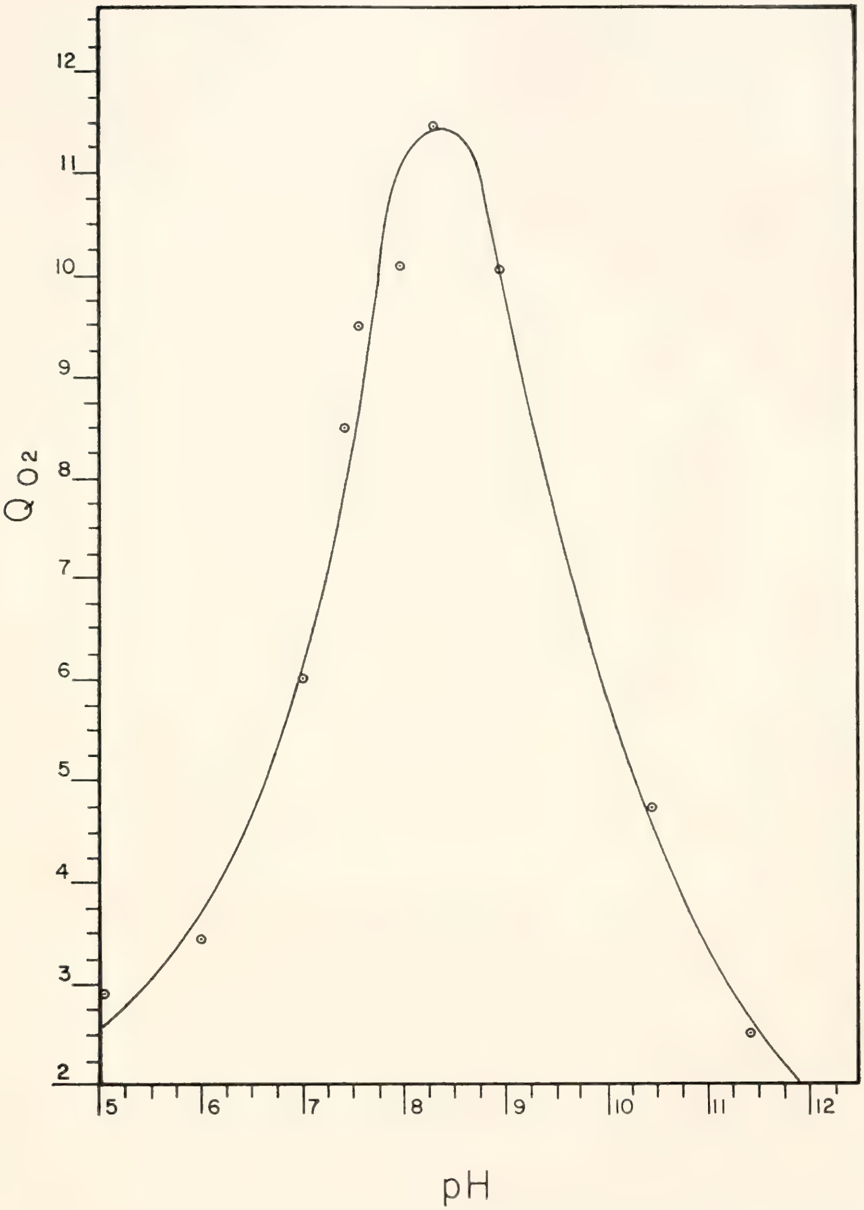


FIGURE 1. The effect of pH on succinoxidase activity in whole body homogenates of *Dugesia dorotocephala.*

in terms of optimum conditions for enzyme activity would be expected at the cellular level. It is possible, too, that the ability of planarians to convert their external medium to a constant pH may be correlated with the optimum activity of succinoxidase in these animals.

Figure 2 shows the direct relationship between concentrations of the homogenate and activity. Since the rate of an enzymatically controlled reaction should increase proportionately to the concentration of the enzyme, in the presence of

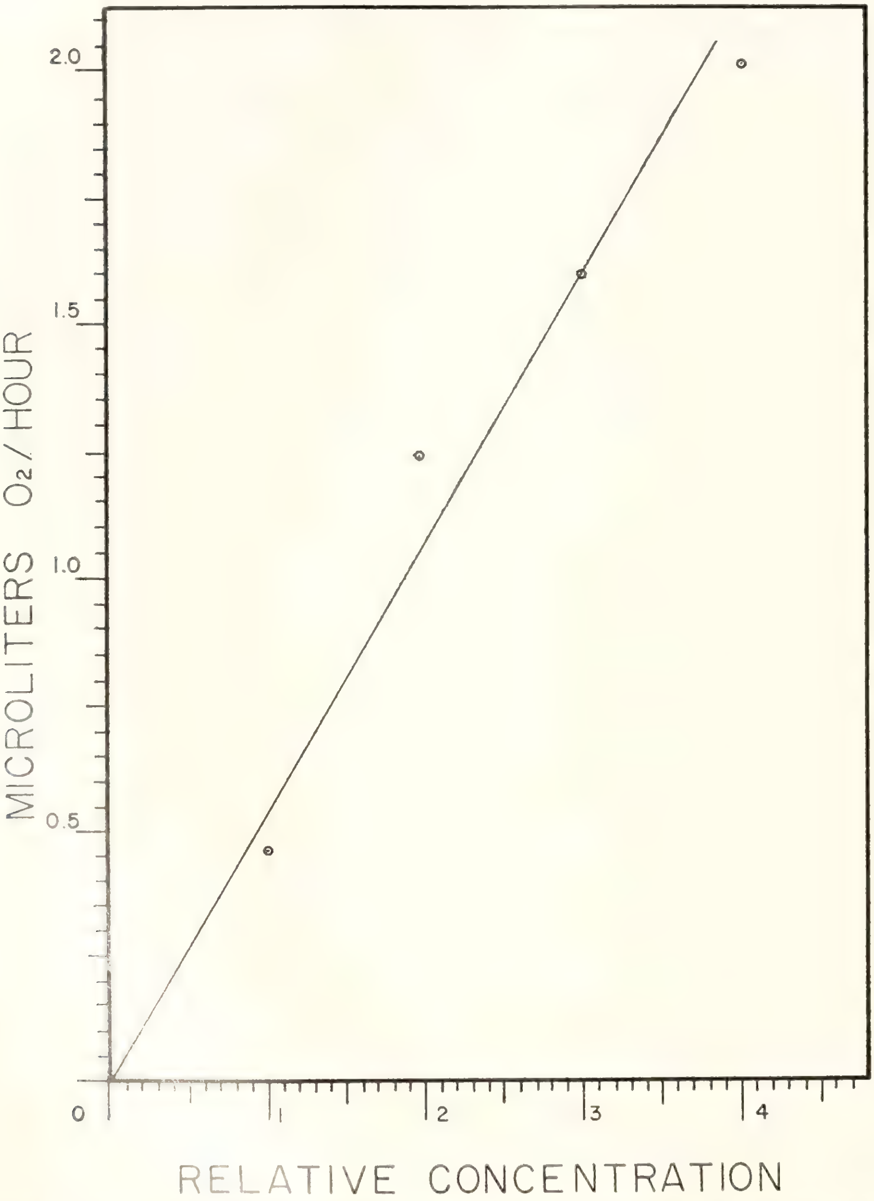


FIGURE 2. Relationship between relative concentrations of homogenate and enzyme activity expressed in oxygen uptake during a 60-minute interval.

excess substrate, it is felt that these results further substantiate the concept of aerobic enzyme systems being active in this invertebrate group.

The effects of adding KCN and urethane to homogenates assayed for succinoxidase activity are shown in Figure 3. The inhibitory results obtained are the

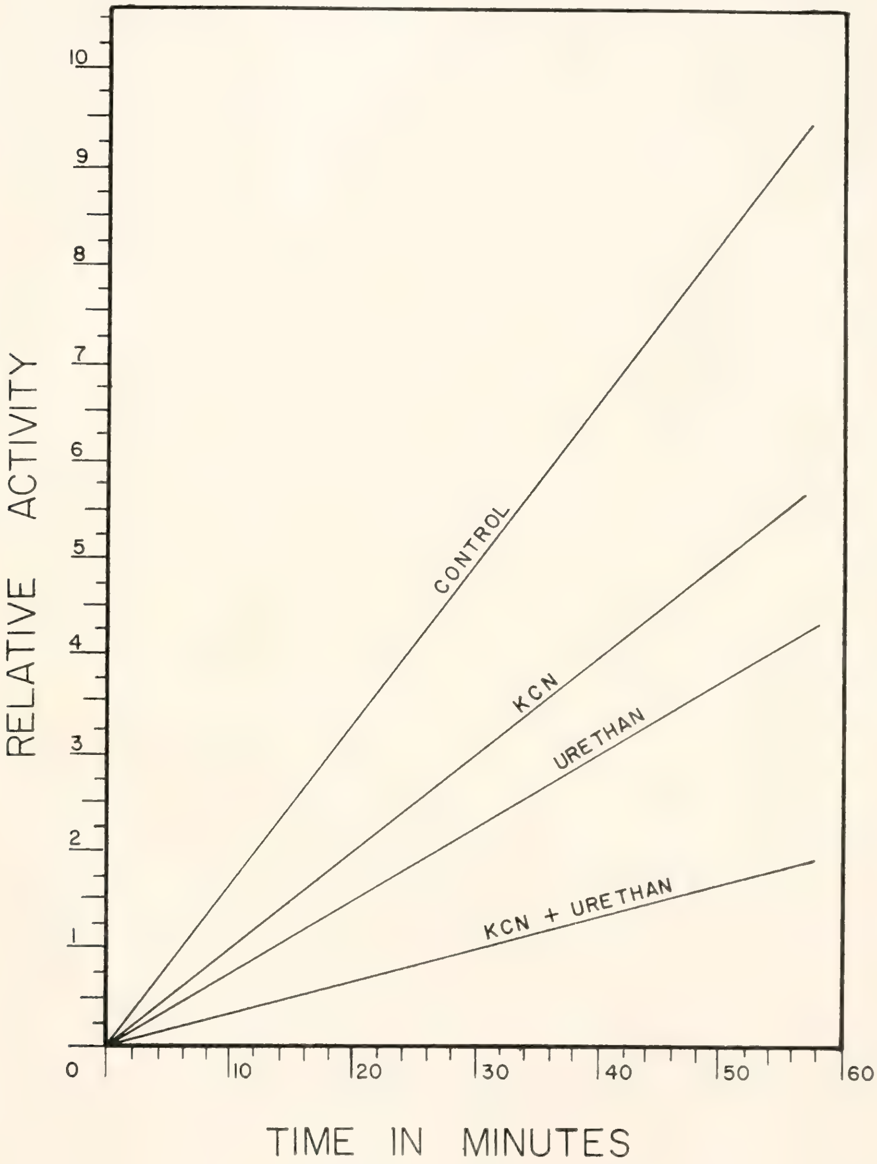


FIGURE 3. Inhibitory effect of 2×10^{-3} *M* KCN and 5×10^{-3} *M* urethane on succinoxidase activity in whole body homogenates of *Dugesia dorotocephala*.

[illegible] would be expected from vertebrate homogenates. Since KCN is a specific for cytochrome oxidase inhibition, and dehydrogenases are considered to be most sensitive to urethane, these results further emphasize the presence of aerobic metabolism.

The evidence presented does not show that all systems or regions of these animals carry on aerobic respiration at the same rate, nor does it imply that both the glycolytic and citric acid cycles are present in all cells at all times. It is apparent that data correlating enzyme activity with regeneration of various regions, and with changes in physiologic and developmental states in *Dugesia,* are desirable. The results reported, however, support completely the indirect evidence for aerobic respiration in planarians quoted previously, and show unequivocally that the succinoxidase system is present in at least some cells of *Dugesia dorotocephala.*

SUMMARY

1. The presence of succinoxidase activity in homogenates of *Dugesia dorotocephala* was investigated, utilizing the Warburg technique.

2. Succinoxidase activity was found to be greater in fed animals than in those starved for 48 hours.

3. Enzyme activity was found to be proportional to enzyme concentration; the activity of the enzyme was inhibited by the addition of KCN and urethane.

4. Optimum enzyme activity occurred at a pH of 8.3, a value considerably higher than the figure quoted for vertebrate tissue.

LITERATURE CITED

Akov, S., and K. Guggenheim, 1963. Antimetabolites in the nutrition of *Aedes aegypti* L. larvae. Nicotinic acid analogues. *Biochem. J.*, **88**: 182–187.

Allen, G. D., 1919. Quantitative studies on the rate of respiratory metabolism in planaria. II. The rate of oxygen consumption during starvation, feeding, growth, and regeneration in relation to the method of susceptibility to potassium cyanide as a measure of rate of metabolism. *Amer. J. Physiol.*, **49**: 420–473.

Bliss, Dorothy E., and Dorothy M. Skinner, 1963. Tissue Respiration in Invertebrates. The American Museum of Natural History, New York.

Bolen, H. R., 1937. Specific dynamic action in planaria. *J. Exp. Zool.*, **75**: 389–412.

von Brand, Th., 1936. Studies on the carbohydrate metabolism in planarians. *Physiol. Zoöl.*, **9**: 530–541.

Ghiretti-Magaldi, Anna, A. Giuditta and F. Ghiretti, 1958. Pathways of terminal respiration in marine invertebrates. I. The respiratory system in cephalopods. *J. Cell. Comp. Physiol.*, **52**: 389–430.

Hammen, C. S., and S. C. Lum, 1962. Carbon dioxide fixation in marine invertebrates. III. The main pathway in flatworms. *J. Biol. Chem.*, **237**: 2419–2422.

Handbook of Biological Data, 1956. W. B. Saunders Company, Philadelphia, Pa.

Hyman, Libbie H., 1919. Physiological studies on planaria. I. Oxygen consumption in relation to feeding and starvation. *Amer. J. Physiol.*, **49**: 377–402.

Hyman, Libbie H., 1951. The Invertebrates: Platyhelminthes and Rhynchocoela. Vol. II. McGraw-Hill Book Company, Inc., New York.

Jenkins, Marie M., 1960. Respiration rates in planarians. I. The use of the Warburg respirometer in determining oxygen consumption. *Proc. Okla. Acad. Sci.*, **40** (1959): 35–40.

LUDWIG, DANIEL, AND MARY C. BARSA, 1956. Oxygen consumption of whole insects and insect homogenates. *Biol. Bull.*, **110** : 77–82.

NIELSEN, C. OVERGAARD, 1961. Respiratory metabolism of some populations of enchytraeid worms and free living nematodes. *Oikos*, **12** : 17–35.

SCHNEIDER, W. C., AND V. R. POTTER, 1943. The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase, and cytochrome oxidase. *J. Biol. Chem.*, **149** : 217–227.

SMITH, AGNES A., AND C. S. HAMMEN, 1963. Effects of metabolic inhibitors on planarian regeneration. *Biol. Bull.*, **125** : 534–541.

STEINBACH, H. BURR, 1962. Ionic and water balance of planarians. *Biol. Bull.*, **122** : 310–319.

THE MECHANICS OF COPULATION IN AEDES AEGYPTI[1]

ANDREW SPIELMAN[2]

Department of Tropical Public Health, Harvard School of Public Health,
Boston 15, Massachusetts

Although the sclerites associated with the genitalia of mosquitoes have frequently been illustrated and form the base for the classification of these insects, their functions are incompletely understood. Several published reports describe the act of copulation in mosquitoes, but none record the precise manner in which the aedeagus is mobilized. Russell and Mohan (1939) fixed copulating pairs of *Anopheles stephensi* and observed that the male claspers engaged the base of one of the terminal abdominal segments of the female rather than the cerci, while the opposing action of the claspettes secured the female ventrally. In contrast, *Deinocerites* males employed their enormously elongated ninth tergite lobes for holding the female (Komp, 1956) and apparently did not use their relatively small claspers. Neither study recorded observations of the means of sperm induction. Rees and Onishi (1951) stated that the aedeagus of *Culiseta inornata* moved "in and out" in a relatively straight line and indicated that it was thereby directed deep into the common oviduct. However, they did not recognize the copulatory bursa of the culicine female (Brelje, 1924) and did not report direct observations of the aedeagus during copulation. Wheeler and Jones (1960) observed that the aedeagus "moves in a ventrally directed arc" in *A. aegypti* but did not report its actual position in coitus. The claspers are apparently the primary holdfast organs in both *A. aegypti* and *C. inornata*, the distal segment being depressed over the cerci of the female.

The present report describes the mechanism through which seminal material is transferred from the *A. aegypti* male to the female. The anatomy of the genital structures is re-described in light of these studies and the course of seminal fluid from the seminal vesicle of the male to the spermatheca of the female traced.

MATERIALS AND METHODS

Specimens were derived from a Johns Hopkins University colony of *A. aegypti* that has been maintained in laboratory culture for more than 20 years. Larvae were reared, using conventional techniques, and pupae were separated as to sex on the basis of size. They were then isolated in individual test tubes where emergence occurred. Adults were subsequently held without food for three to four days.

---

[1] This study [illegible] part by Public Health Service grant No. 5 T1-AI-46 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[2] I thank Dr. Jack Colvard Jones of the University of Maryland for his critical reading of this manuscript and for his cooperation during the course of this study. The illustrations were prepared by Miss Fran [illegible] of Brookline, Massachusetts.

Pairs were permitted to copulate naturally by introducing virgin females into standard lantern chimneys containing 15 to 20 males. Their activity was observed and the duration of genital contact recorded. Alternatively, copulation of restrained pairs was induced using a modification of the McDaniel and Horsfall (1957) technique. Nitrogen was used to anesthetize males and ether to anesthetize females. Carbon dioxide anesthesia appeared to interfere with copulation.

Mosquitoes in various stages of copulation, as well as individual specimens, were killed by freezing. They were taken from the lantern chimneys by aspirator tube and quickly transferred to a freezing chamber immersed in an alcohol-solid CO_2 bath. Copulating pairs retained genital contact throughout this process. Subsequently, material was studied in one of several ways. While still frozen, they were fixed in Newcomer's solution (Newcomer, 1953) at solid CO_2 temperature. Some were transferred to 95% alcohol and then to beechwood creosote for dissection. Permanent mounts of 73 males and 36 females were prepared in this manner. Other specimens were imbedded after fixation, sectioned serially at 6–8 μ and stained with hematoxylin-azure II-eosin. Thirty-eight females and 15 males were studied in this manner. More than 20 additional specimens were prepared in the conventional manner by maceration in KOH and staining with acid-fuchsin. Supplemental observations were made with more than 100 males and females that were freshly dissected in *Drosophila* Ringer's solution.

OBSERVATIONS

Behavior patterns preceding copulation. Males attempted to attach themselves to flying females or to a tuning fork simulating the sound of the female's wings, as described by Roth (1948). When males established contact with a female whose wings were in motion, they tended to retain their hold even after wing motion had ceased. However, males did not retain contact with females whose wings were at rest when contact was established. Twenty 4-day-old virgin females, whose wings had been removed, were placed in a lantern chimney with 20 virgin males of the same age. None was inseminated after 24 hours. The experiment was repeated, but with one-half of the females wingless. Only the winged females (10 out of 10) were inseminated during the 24-hour exposure period. The initial contact was therefore established chiefly during flight, and this appeared to be the invariable rule when copulation was directly observed.

Genital contact, however, rarely occurred during flight in the small mating chambers. More than 200 copulating pairs were systematically observed and only in three were the abdomens in contact when they landed upon the container's floor. The remainder flew about momentarily after pairing and generally landed upon the floor with the male's tarsi in contact with the female's. Thereupon, the males made strenuous attempts to invert themselves beneath the females, if they were not already in this position, while arching their abdomens and moving their claspers in a grasping manner. The females' wings were not in motion while this occurred. Copulation was aborted about half the time as males attempted to invert themselves. Once tarsal contact was lost it was never regained while the pair was at rest. When a copulating pair was disturbed, the mosquitoes frequently flew off with their genitalia in contact and assumed the mating postures described by Roth (1948).

Whole-mount preparations of male terminalia.

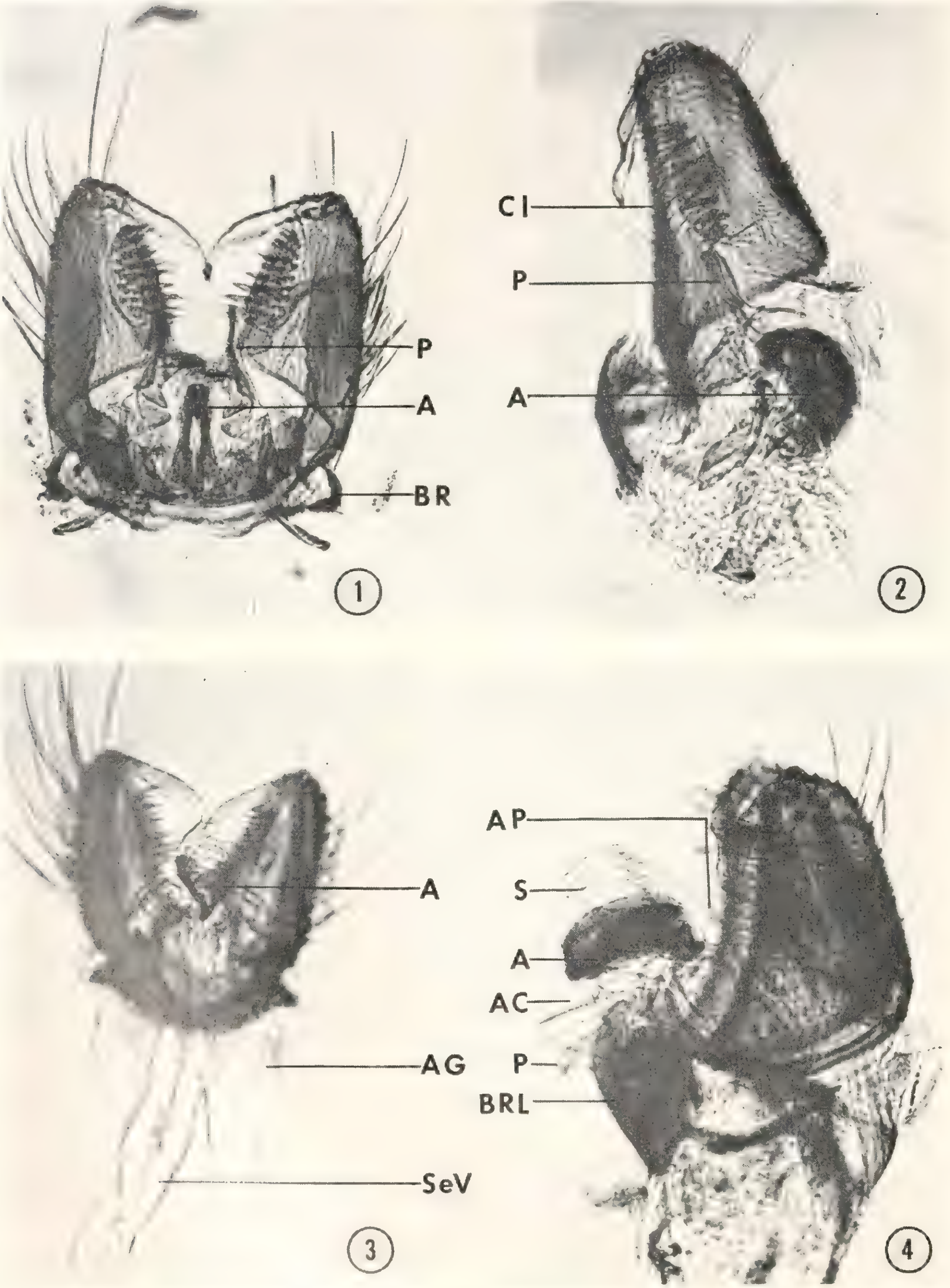

P
A
BR
1
Cl
P
A
2
A
AG
SeV
3
AP
S
A
AC
P
BRL
4

Figure 1. Ventral aspect of non-copulating male, showing relationship of paraprocts (P) to aedeagus (A) and position of basal ring (BR).

The cuticular sheet from which the clasper is derived is partially rolled into a cylinder. Its apodeme is continuous with the body of the clasper but is rotated so as to reverse its dorso-ventral orientation (illustrated by Christophers, 1960, but not by Hodapp and Jones, 1961). The dorsal root of the apodeme bears a thickened ridge upon the ventral surface of which the apodeme of the aedeagus articulates. The basal segment is inserted in the membranous area closing each clasper's cylindrical wall, and is continuous with the bridge connecting it with the basal segment in the opposite clasper. These segments are thickly studded with strong setae and each bears two or three prominent setae whose ends are finely tapered and bent. These setae, apparently specialized sensillae, are innervated from the last abdominal ganglion. The distal lobe is rod-like, and, at its end, bears a socketed claw.

The copulatory apparatus of the male is supported by the basal ring which is derived from the combined plates of the ninth abdominal segment. The dorsal portion of the ring appears to be sessile in relation to the preceding segment. The ventral portion, however, articulates with the dorsal at a point just distal to the base of the prominent ventral lobes. These large, cup-like lobes shield the anus on its ventral surface.

Two membranous structures arise from within the basal ring and envelop the aedeagus and paraprocts (Figs. 4, 7). Ventrally, the anal cone (proctiger) is continuous with the inner margin of the lobes of the basal ring and with the dorsal surface of the anus. This prominent, cone-shaped structure is continuous on its dorsal surface with the ventral margins of the aedeagus and of the claspers. In ventral view, the anal cone obscures the aedeagus and all but the hooked projections of the paraprocts. The lateral lobes of the paraprocts are inserted on its dorsal surface. The aedeagal pouch arises dorsally from the bridge that connects the clasper's basal lobes, and is continuous with the base of the aedeagus on its ventral margin. This membranous ridge shields the dorsal aspect of the aedeagus and paraprocts.

Seven paired muscles associated with the sclerotized portions of the copulatory apparatus are illustrated in Figure 8. They include: (1) a small muscle which extends the distal segment of the clasper; (2) a muscle that retracts the distal lobe of the clasper against the basal lobe; (3) a very heavy muscle which retracts the clasper apodeme against the body of the clasper; (4) a prominent group of muscle fibers which connects the clasper and the dorsal portion of the basal ring and serves to extend the entire copulatory apparatus dorsally; (5) a relatively small muscle that retracts the apodeme of the aedeagus against the distal portion of the apodeme of the clasper; (6) a long, slender muscle that retracts the lateral segment of the paraproct against the apodeme of the clasper; and (7) a muscle that draws the apodeme of the aedeagus against the body of the clasper.

FIGURE 2. Lateral aspect.

FIGURE 3. Ventral aspect of copulating male showing everted aedeagus and positions of seminal vesicles (SeV) and accessory glands (AG). Note constriction in accessory gland of this ejaculating male and presence of ejected seminal material.

FIGURE 4. Lateral view showing ejected seminal material (S), position of paraprocts during copulation and position of anal cone (AC) and everted aedeagal pouch (AP).

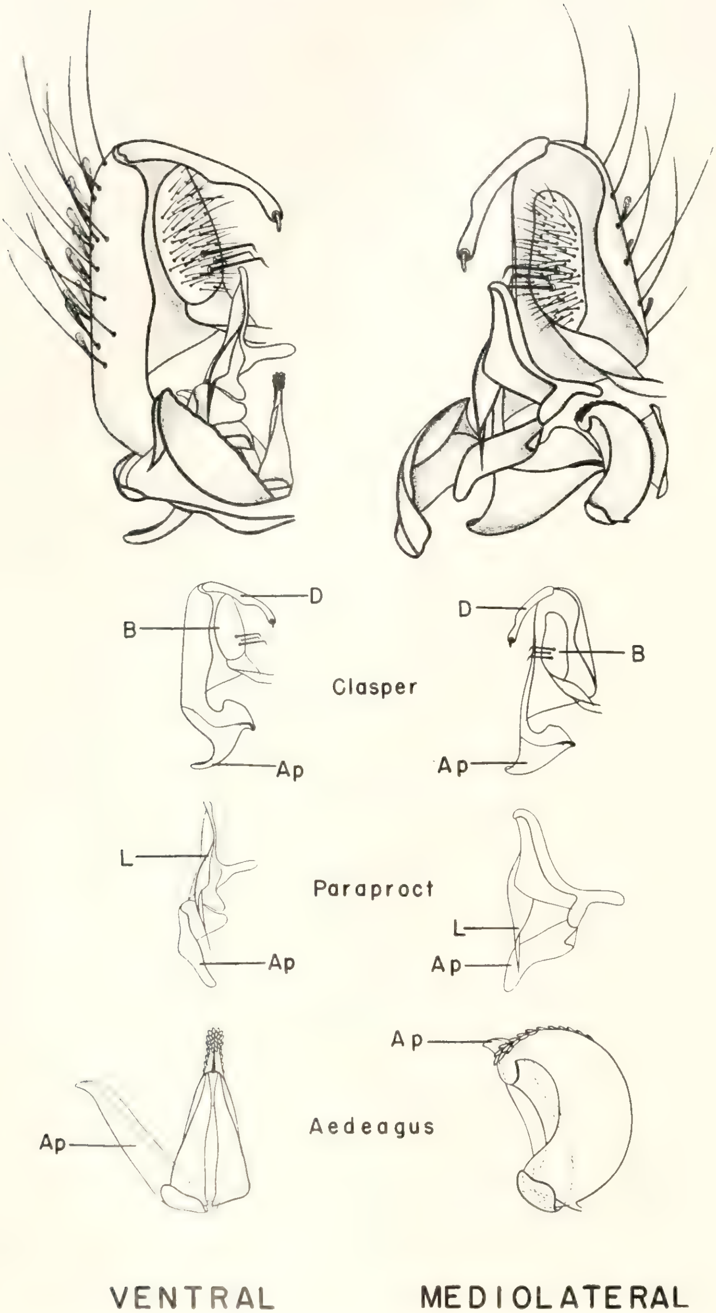

D
B
Clasper
Ap
D
B
Ap
L
Paraproct
Ap
L
Ap
Ap
Aedeagus
Ap
VENTRAL
MEDIOLATERAL

Figure 5. Terminalic sclerites of non-copulating male, hemisected and with functional components separated. (Corresponds to Figures 1, 2.)

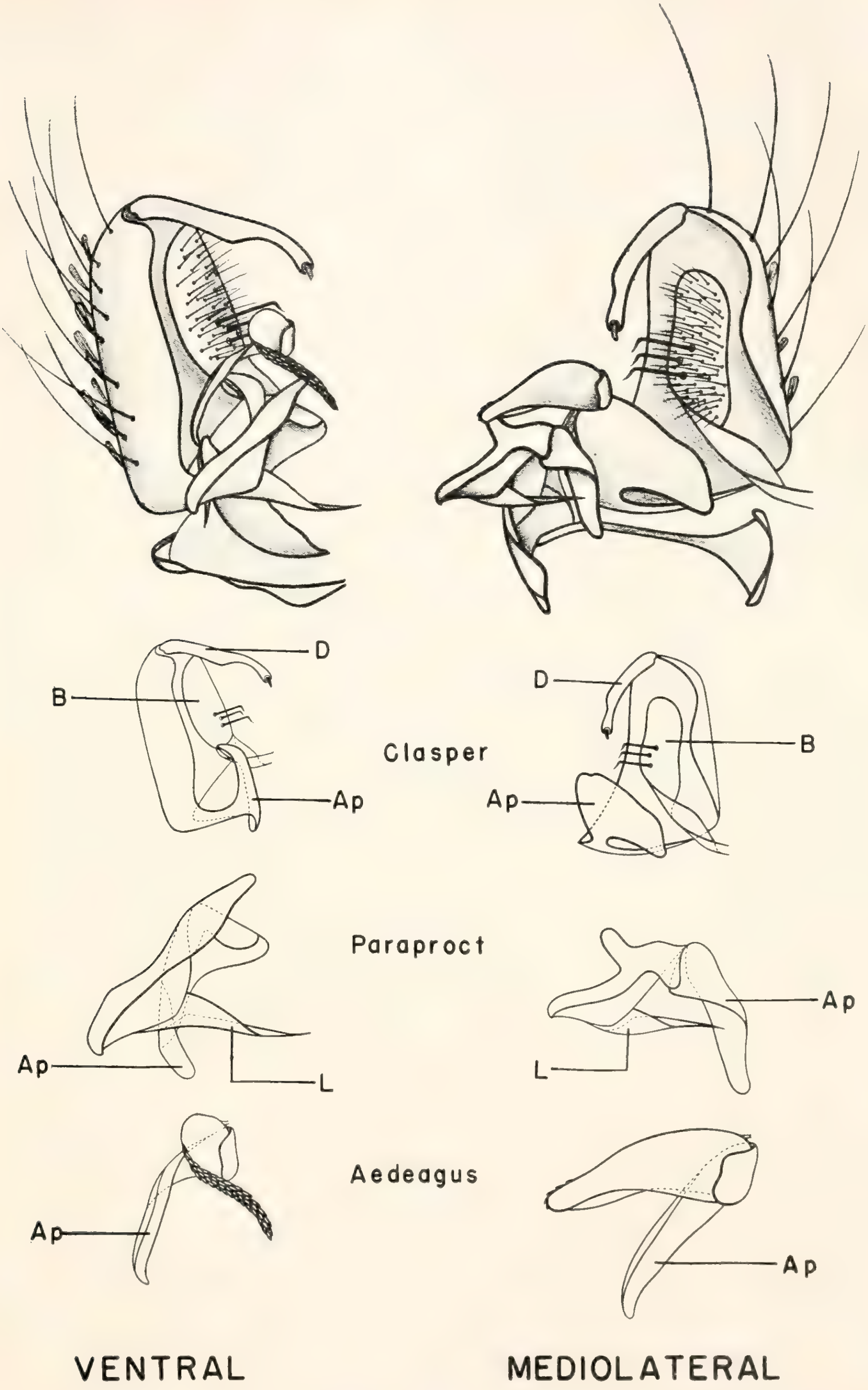

D
B
Ap
Clasper
D
B
Ap
Paraproct
Ap
Ap
L
L
Aedeagus
Ap
Ap
VENTRAL
MEDIOLATERAL

FIGURE 6. Terminalic sclerites of copulating male, hemisected and with functional components separated. (Corresponds to Figures 3, 4.)

The gross morphology of the internal copulatory structures of the male has been described by Christophers (1960), Hodapp and Jones (1961) and by Lum (1961). The following observations amplify these descriptions. The ejaculatory duct leads directly into the genital pore which is located near the base of the aedeagus (Fig. 7). The duct wall is composed of three layers of tissue: an innermost layer of thin cuticle, a low epithelium, and a thin overlying syncytium of transverse muscle fibers. When at rest the duct is contracted and virtually closed, and no internal movements were noted when it was dissected free of surrounding tissue. The paired accessory glands arise near the anterior end of the ejaculatory duct. The posterior portion of each gland is filled with a granular material which appears to be derived from disintegrating cells filling the anterior part of the gland and lining its walls. A syncytium of transverse muscle fibers overlying these glands provides them with an effective means of contraction. Deep, slow, nonprogressive contractions were observed repeatedly. The paired seminal vesicles are continuous with the anterior end of the ejaculatory duct and are packed with masses of quiescent sperm, the heads of which are directed against the opening of the ejaculatory duct. An unlined low epithelium with an overlying spiral layer

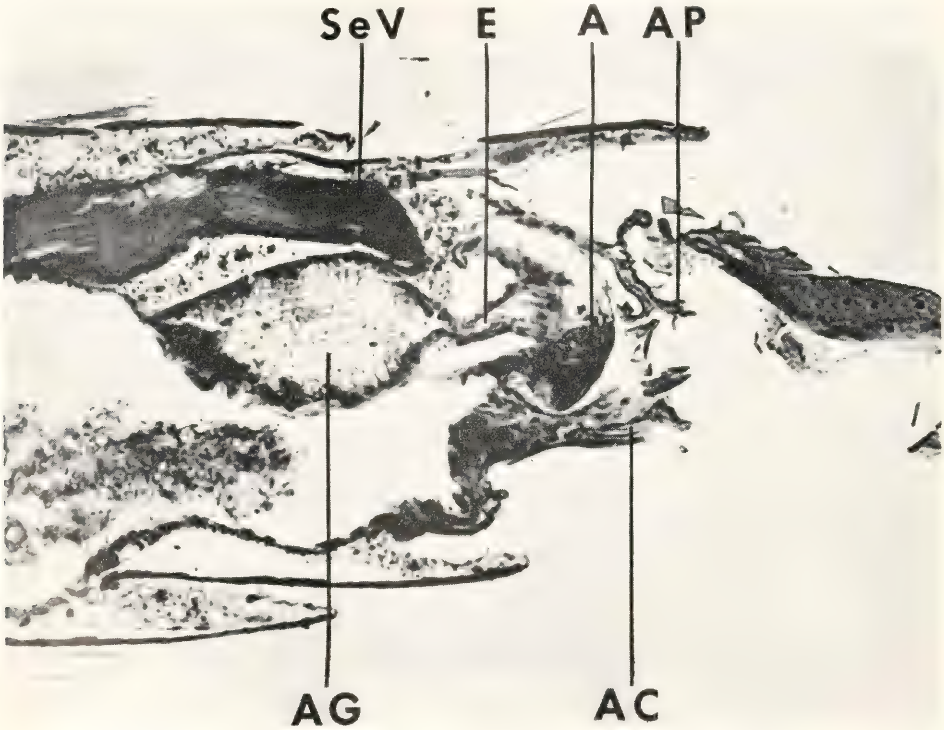


FIGURE 7. Sagittal section of non-copulating male, showing insertion of ejaculatory duct (E) in the aedeagus and placement of aedeagus between aedeagal pouch (AP) and anal cone (AC).

of muscle fibers comprises the wall of this organ. Lashing motions and nonprogressive point contractions were visible in dissected seminal vesicles.

Changes occurring in the male copulatory apparatus during copulation. A profound change in the appearance of the male copulatory apparatus results when the flexor of the apodeme of the clasper contracts (Figs. 1–6). This movement initiates a chain of events that culminates in the erection of the aedeagus. The flexor rotates the ventral portion of the clasper apodeme dorsally and posteriorly and brings it in apposition to the body of the clasper. Thus, the apodeme, which is in a twisted position when the male is at rest, becomes straightened in copula and folded back toward the medial surface of the clasper. This process results

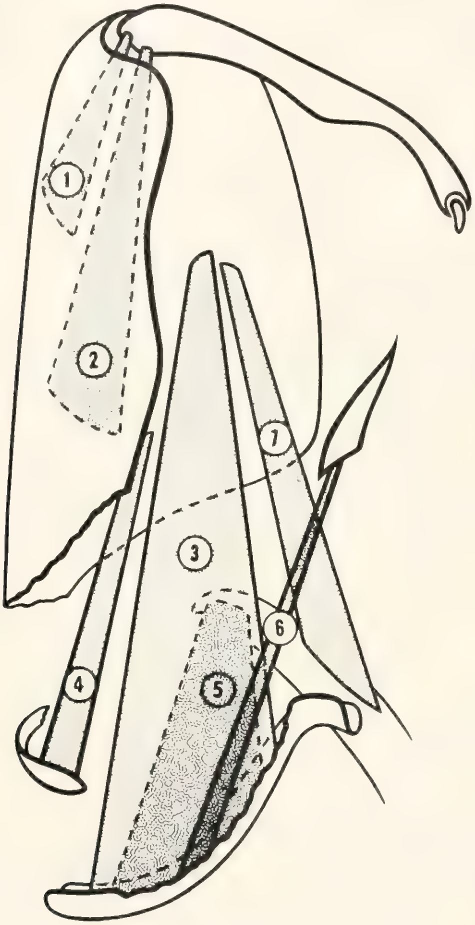
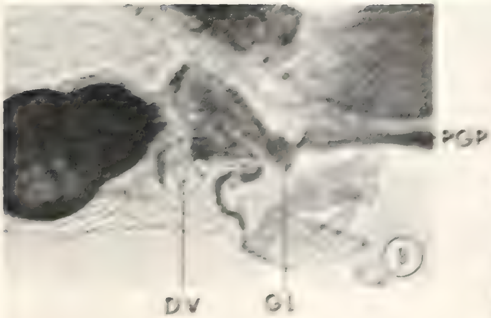


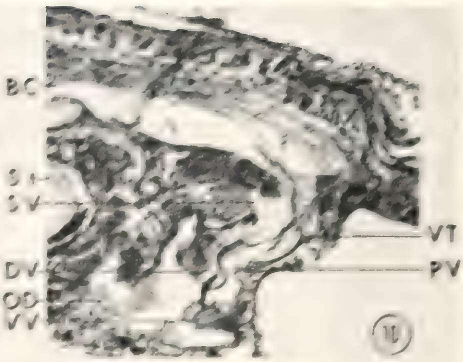
FIGURE 8. Diagram of the muscles of the male genital sclerites. 1, extensor of distal segment of clasper; 2, flexor of distal segment; 3, flexor of apodeme of clasper; 4, extensor of clasper; 5, flexor of apodeme of aedeagus; 6, flexor of lateral segment of paraproct; 7, extensor of apodeme of aedeagus.

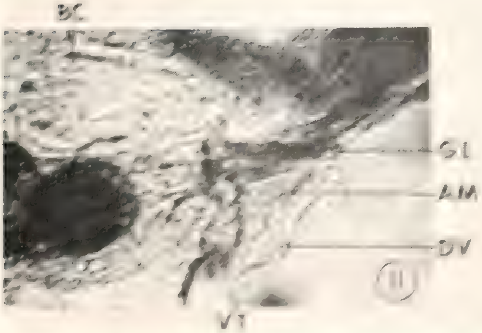
[illegible]

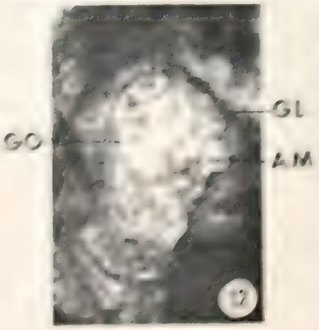
[illegible]

[illegible]


PGP
DV
GI
9


BC
S
SV
DV
OD
VV
VT
PV
10


BC
GI
AM
DV
VT
11


GL
GO
AM
12

[illegible]

[illegible]

[illegible]

[illegible]

pory. In section, the inner aedeagus is roughly heart-shaped, and is armed with rows of strong teeth along its distal margin.

The eversion and extrusion of the aedeagus results in a radical change in the position of the paraprocts. The lateral cone-like projections, which lie in close proximity to the aedeagus, is the lever by which the body of the paraproct is moved. The paraproct hinges on its apodeme and extends in a ventro-lateral direction due to pressure exerted against the cone by the aedeagus. The hook-like extension of the paraproct is thereby projected toward the lobes of the basal ring while the apodeme of the paraprocts remains virtually stationary. The basal segment is drawn toward the apodeme of the clasper by its flexor (muscle 9, Fig. 9), which serves to steady the extended paraprocts and to draw the hooks tightly against the ventral surface of the inner aedeagus.

Less radical changes occur in the position of other structures during copulation. The retraction of the distal lobe of the clasper draws that structure toward the basal plate of the clasper (muscle 2, Fig. 9). At the same time, the position of the claspers is shifted dorsally by contractions of the muscles connecting them to the basal ring. The ventral lobes of the basal ring are connected to the apodemes of the three genital sclerites, and these structures tend to rotate ventrally.

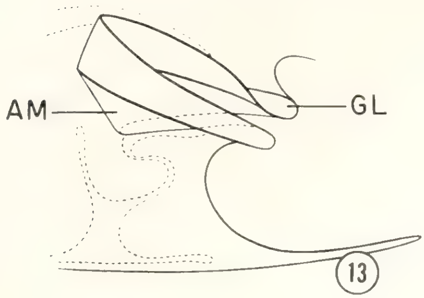
Description of the female copulatory apparatus. The genital opening of the female is guarded by the genital lips, a circular ribbon-like plate which is folded upon itself and articulates at the fold (Figs. 9–11). The lower lip bears an external extension, known as the insula, while internally a tuft of hairs, the ventral lobe, projects from the lip's dorsal edge. A transparent membrane, the atrial membrane, is continuous with the lower margin of the lips and includes a pair of sclerotized plates, the atrial plates (Figs. 15, 16). At rest, the genital lips are in contact, with the upper lip extending somewhat beyond the lower and with the atrial membrane infolded.

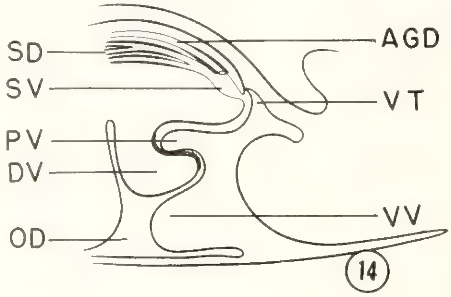
The genital lips are continuous with the posteriorly projecting postgenital plate, and are concealed by the shell-like abdominal sternites. The postgenital plate, which separates the genital and anal openings, bears an inferiorly elongated groove on its ventral surface. The setae on the upper genital lip form a channel with this groove. The internal aspect of the groove serves as the insertion for the series of retracting muscles that control the movements of the post-genital plate, cerci and upper genital lip.

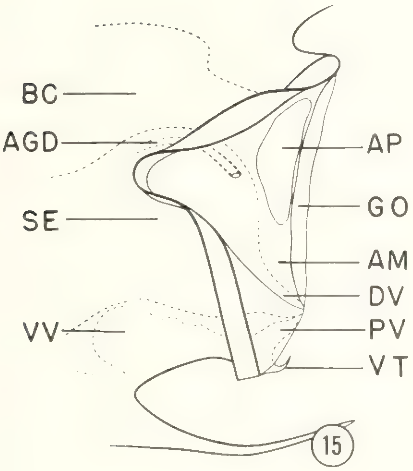
The internal organs of the copulatory apparatus of the female communicate with each other and with the genital opening through the completely folded genital atrium (Fig. 14). The atrium has four openings: dorsally, it communicates with the copulatory bursa; ventrally with the common oviduct; antero-dorsally with the spermathecal ducts; and postero-ventrally with the genital lips. The thin layer of cuticle that lines the upper portion of the atrium is smooth. The thickened lower portion of the atrium is lined by a relatively heavy cuticle bearing many rows of tubular hairs just below the junction of the spermathecal ducts. A layer of low epithelial cells underlies the epithelial covering of the entire atrium. A series of three valve-like structures closes the lower atrium and appears to prevent the opening of the common oviduct. The dorsal and ventral valves oppose each other and form the limiting lines of the lower atrium. The posterior valve serves to close the lower atrium, and, with the dorsal and ventral valves, guard the lumen of the oviduct.

The copulatory bursa is a blind sack that arises just internal to the upper genital lip. In virgin females, it is filled with an amorphous eosinophilic material and is composed of a thin layer of squamous epithelium with a light cuticular lining. The bursa extends anteriorly beyond the spermathecae where it is flattened dorsoventrally and folded upon itself. No contractions were seen in this structure during the course of the study and no muscles or nerves were found.

Female terminalia (corresponds to Figures 9–12).







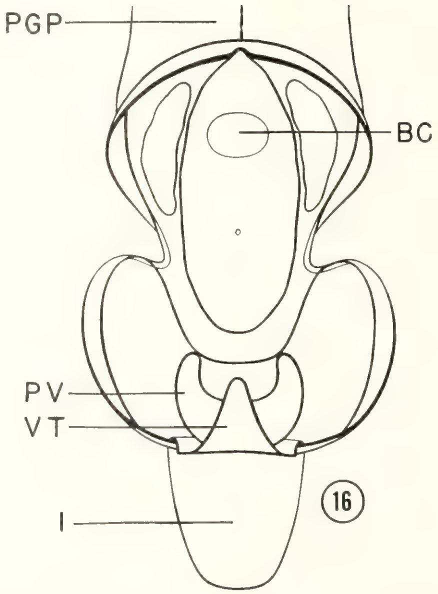


Figure 13. Lateral view of non-copulating female, showing infolding of atrial membrane (AM).

Figure 14. Sagittal section, showing structure of the atrium and spermathecal vestibule (SV).

Figure 15. Lateral aspect of copulating female, showing relationship of genital orifice (GO) to genital lips and posture of spermathecal eminence (SE) during coitus.

Figure 16. Caudal view, showing structure of extended atrial membrane.

The spermathecal eminence is a mound of fibrous connective tissue and transverse muscle fibers lying between the copulatory bursa and the common oviduct. A shallow, vertical groove marks its upper surface and terminates in a prominent cavity, the spermathecal vestibule (Figs. 10, 14). The accessory gland duct and the three spermathecal ducts communicate with the atrium through this cavity. The ducts of the two lateral spermathecae anastomose first. The duct of the medial spermatheca and the common duct of the lateral spermatheca fuse with the anterior wall of the vestibule, while the heavily cuticular accessory gland duct fuses with its mid-dorsal wall. The vestibule has no distinct lining and no muscles or valves are associated with its lumen. Its opening on the posterior-most surface of the spermathecal eminence appears to be guarded by the ventral tuft. Spasmodic twitching movements of the eminence are occasionally seen in fresh preparations and are apparently due to contractions of the transverse muscle bundle.

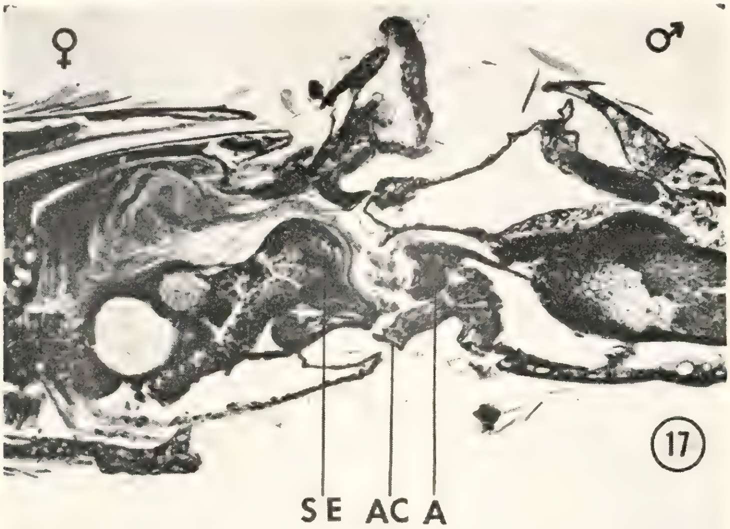
The three spherical spermathecae lie at the anterior end of segment VIII between the common oviduct and the copulatory bursa, and are partially embedded in a dense mass of visceral fat. The median spermatheca is the larger and the more anterior of the two lateral spermathecae. Each is a darkly pigmented, heavily cuticular, capsule which is naked on its inner, luminal surface. A series of minute pores is present near the orifice of each theca but not beyond the posterior third. Externally, a secretory cell is contiguous with each pore and surrounds a minute flask-shaped projection of the pore's rim. The nuclei and cytoplasmic granules of these cells are located in the portion of the cell away from the lumen of each spermatheca, and secretory vacuoles are sometimes present in the proximal portion of each cell. A thin squamous epithelium surrounds the remainder of the outer surface of the spermathecae. An extended constriction of the spermathecal capsule connects with the colorless layer of cuticle that lines the spermathecal ducts.

The spermathecal ducts are covered throughout their length by a thin epithelium. They emerge from the eminence anterior to the bifurcation of the duct of the smaller spermathecae. This anterior portion is covered by a layer of muscle tissue and an overlying membrane. Large cells, containing secretory vacuoles, are attached to the free portion of the ducts. They resemble the spermathecal gland cells, but are pedunculate and are attached to the spermathecal ducts by a narrow extension of cytoplasm. A fine, cuticle-lined capsule is present within each cell in an eosinophilic, non-granular, portion of the cytoplasm. Secretory vacuoles form in this region, but neither the capsule nor the vacuoles have been traced to the lumina of the ducts.

Description of the process of copulation. When abdominal contact is first established, the male's claspers engage the cerci of the female (Figs. 17–19). This brings the basal lobes of the claspers into contact with the female's post-genital plate. Specifically, the prominent hairs with recurved tips are directed into the groove on the ventral aspect of the post-genital plate. Following this contact, the aedeagus everts. Naturally-copulating pairs were observed repeatedly under conditions permitting recognition of the aedeagus if it were everted, but this was never seen unless the cerci were engaged. Experience with forced copulation confirms the observation that aedeagal eversion follows the establishment of abdominal contact.

As the aedeagus is everted, the apices of the paraprocts are extended and contact the lower genital lip of the female. Continued pressure by the aedeagus through the paraprocts forces the lip ventrally. Thus, the opposing action of the basal lobes of the claspers and the paraprocts deforms the genital lips of the female. Eventually, the paraprocts come to rest with their cam-like projections

Photomicrographs of copulating pairs in lateral aspect.



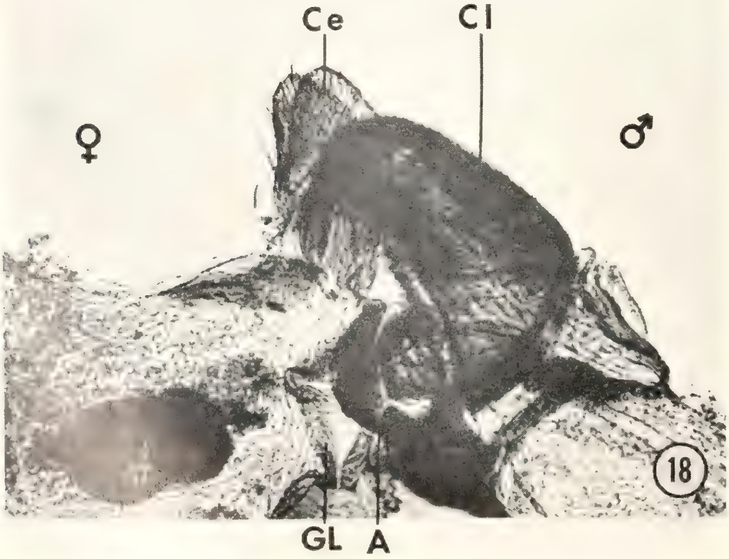


FIGURE 17. Sagittal section, showing continuous channel formed by everted aedeagus and extended atrium of the female.

FIGURE 18. Whole-mount preparation, showing attachment of claspers to cerci of female and position of everted aedeagus relative to the genital lips.

placed in the folded hinge of the lips and with the hooked apices against the lower genital lip (Fig. 19).

The female's terminalia have now undergone a radical change in shape (Figs. 15, 16). The atrial membrane is everted when the genital lips are extended and the genital orifice is exposed. The orifice itself is at the end of this membranous, sleeve-like extension of the genital lips and precisely matches the outline of the everted aedeagus' dorsal extended surface. The most distal margin of the aedeagus is placed just inside the female genital orifice, but was never observed to be inserted even as far as the genital lips. The position of the aedeagus varied relative to the female, contact being established at any point along its outer margin.

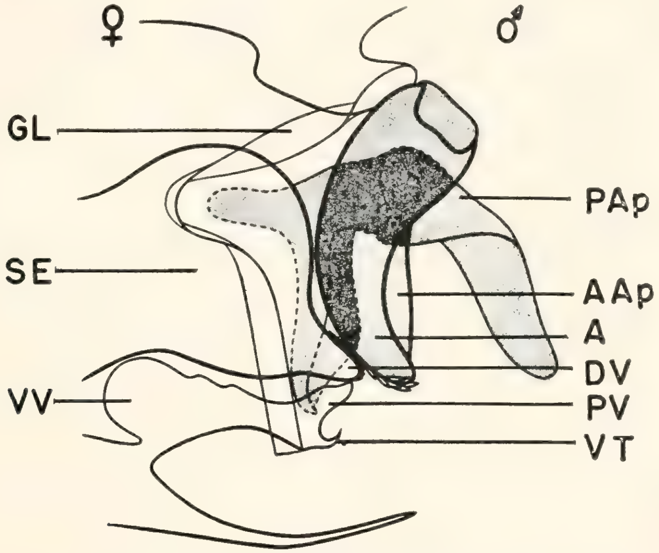


Figure 19. Position of aedeagus (A) and paraprocts (P) (both shaded) relative to the female during copulation (hemisected).

A firm seal is established between the extended genital parts of the copulating pair (Fig. 17). Laterally, the seal is formed by the spread lobes of the aedeagus within the atrial membrane. The paraprocts press against the female's atrial plates and in this manner serve to complete the lateral seal. Dorsally, the aedeagal pouch closes the space between the aedeagus and the upper genital lip, while ventrally, the seal is completed by the anal cone. The lateral lobes of the paraprocts stretch the anal cone against the aedeagus as the paraprocts are spread. The pair is thus joined in copula.

The shape of the female's atrium is disrupted as the atrial lips are distended. Most notably, the dorsal valve is drawn posteriorly and the posterior valve and ventral tuft are everted. The spermathecal eminence assumes a "goose head" appearance with the dorsal valve representing the beak. The spines on the dorsal surface of the valve are thereby exposed to the aedeagus of the copulating male. When in copula, the aedeagus lies against the outer surface of the dorsal valve with its ventrally projecting spines engaging the dorsally projecting spines of the [illegible]

The mechanism of copulation may be represented by the following model. The male presents to the female the base of the cup-like aedeagus containing an ejaculatory apparatus in its vertex. The copulatory bursa of the female is extended to form an elongated blind pouch. These two structures are appressed, sealed, and subsequently inflated with seminal material. Deep intromission does not occur.

Transfer of sperm to the copulatory bursa of the female apparently results from contractions occurring in the male accessory glands and seminal vesicles. The ejaculatory duct is weakly muscled and appears to be a simple valve. This duct was fully distended in all ejaculating males sectioned. Deformations of the accessory glands and seminal vesicles, in addition, were observed solely in ejaculating males (Fig. 3). Sperm motility did not appear to play a role in transfer to the female. Sperm were invariably transferred in masses and were layered against the anterior wall of the bursa during copulation. It is suggested that the accessory glands provide the vehicle and the motive power through which the sperm are transported. Accessory gland contractions are strong and material is extruded from them when the integrity of the gland wall is disrupted (Lum, 1961). Contractions occurring in the seminal vesicles have less amplitude and it is suggested that their function is to release bundles of sperm into the streaming accessory gland fluid.

Table II

Location of sperm in A. aegypti *females at intervals after termination of coitus (coitus interrupted after 10 seconds' duration)*

Time after coitus terminated	Number of females	Number of females with sperm in		
		Spermathecal vestibule	Spermathecae	Oviduct
25 sec.	1	0	0	0
30	3	0	0	0
35	2	1	0	0
40	2	2	1	0
45	2	1	1	0
50	2	2	0	0
55	2	2	0	0
60	2	2	2	0
2–5 min.	8	8	8	0
10	2	0	2	0
30	2	0	2	0*
60	2	0	2	2

* Sperm present in lower atrium in both females.

Observations on spermathecal filling. The fate of the seminal mass in the copulatory bursa was studied in females frozen at regular intervals after copulation. Some were sectioned (Table II) and others prepared as whole-mounts. In each freshly inseminated female studied, the copulatory bursa was inflated and the walls greatly distended. During coitus, sperm were deposited in four or five ventrally directed arcs intermixed with a granular gel which appeared to be derived from the male accessory glands (Fig. 17). Subsequently, sperm dispersed through the bursal contents.

The architecture of the atrium and of the atrial valves regained its precopulatory appearance within 30 seconds after termination of coitus. However, during the ensuing hour, the ventral tuft of each specimen continued to be separated from the spermathecal eminence by a space that exceeded the diameter of the vestibule. Subsequently (at about 35 seconds), sperm began to concentrate on the ventral wall of the bursa just above the spermathecal eminence. They were oriented toward the spermathecal vestibule with their heads directed into that structure. This mass of sperm, designated as the sperm plug, was present in each female in which spermathecal filling was observed (Table II). Among these females, sperm first appeared in the spermathecal ducts about 40 seconds after coitus and most females contained some sperm at 60 seconds. At this time, the sperm plug was very dense and contained a mass of cells which appeared to fill the lumen of the vestibule. In general, only a few sperm were seen at any one time in the ducts of sectioned material and these were invariably oriented with their heads toward the spermatheca. Although the accessory gland duct remained open, sperm never appeared to enter that structure.

At 5 minutes after coitus, sperm were scarce in the anterior portion of the copulatory bursa and the spermathecae appeared to be normally filled with sperm. Although the sperm plug was undiminished in size, sperm were rare in the spermathecal ducts. Simultaneously, the granular material contained in the bursa began to swell and to take on a "globular" appearance. This was accompanied by a thickening and vacuolization of the cells of the bursal wall.

The sperm plug was no longer present 10 minutes after copulation, and the remaining sperm appeared concentrated in the upper atrium. Small numbers of sperm were recognized in the lower atrium beneath the level of the spermathecal vestibule at 30 minutes.

At one hour after coitus, the common oviduct contained masses of agglutinated, immobilized sperm. The bursa was filled with coarse material and the remaining sperm were compressed into its posterior end and into the upper atrium. The bursal wall was greatly thickened and much vacuolated and the bursa itself reduced in size. By the next day, the globules had been replaced by amorphous, granular material, and the bursal walls had become squamous. Although motile at this time, bursal sperm lost their motility by the second day after coitus.

Discussion

The aedeagus does not function as a deep intromittent organ in *Aedes aegypti*. Rather, seminal material is transferred to the female through close apposition of genital parts of the copulating pairs. This precise mechanical relationship between the sexes during copulation recalls Dufour's "lock and key" hypothesis. *A. aegypti* and *A. albopictus* are mechanically incompatible (Leahy, 1962), and semen wasted in such matings has been found externally on the abdomens of females. Thus, terminalia of each member of a mosquito population must be of constant form and dimension, a phenomenon that has been exploited by taxonomists for many years.

Intromission occurs during copulation in various Diptera. Male *Phlebotomus* spp. possess a tube-like genital filament that is inserted into the spermathecal ducts of the female (Hertig, 1949). Sperm are thus deposted directly into the spermathecae in these organisms. Anophelines (but not culicines) have a similar filament that lies within the folds of the aedeagus (Hodapp and Jones, 1961). The aedeagus of the copulating *Anopheles stephensi* illustrated by Russell and Mohan (1939) appears to be placed superficially upon the body of the female. Comparable slide material supplied by B. N. Mohan confirmed this impression. Taken together, this suggests that anophelines may copulate in a manner analogous to that of *Phlebotomus,* that is, through deep intromission of a genital filament. Giglioli (1963), however, theorized that sperm of *A. gambiae* are discharged near the orifice of the spermathecal duct and that they subsequently migrate to the spermathecae. Snodgrass (1963) emphasized that the aedeagus need not be regarded as an organ of deep intromission and the present study supports this concept.

In contrast, *A. aegypti* sperm are delivered to the copulatory bursa, and do not transfer to the spermathecae until after the copulating pair has separated. Transfer commences at approximately 40 seconds after copulation and continues for several minutes. Those sperm that fail to transfer to the spermathecae, together with the seminal fluid, are subsequently digested and absorbed in the bursa.

The correct location of the genital openings in *A. aegypti* has not previously been described. It has generally been assumed that the male genital opening is located at the apex of the aedeagus (Hodapp and Jones, 1961; Christophers, 1960), and the female opening was thought to be bordered by the genital lips (Christophers, 1960). The aperture located above the upper genital lip of an *Aedes aegypti* female described and figured by Curtin and Jones (1961) is perhaps an artifact. This study demonstrates that the male genital opening is located at the base of the aedeagus and the female opening at the apex of the everted atrial membrane. Atrial plates were recognized by Coher (1948) in species of a number of culicine genera but not in *Aedes.* However, our observations, as well as those of Burcham (1957), demonstrate that they also occur in *A. aegypti.* The function of these plates must be that of supporting the atrial membrane, and of extending the female genital opening.

While these descriptions of the anatomy of the internal components of the male terminalia are in general agreement with those of Christophers (1960) and of Hodapp and Jones (1961), our observations upon the female differ in several respects from published reports. The arrangement of the atrial chambers and the structure of the atrial membrane have not been previously reported. However, the ventral tuft (vaginal membrane or vaginal valve) was noted by Parks (1955) and Burcham (1957). Leahy (1962) suggested that this tuft was a filtering organ which separated sperm from accessory gland material after termination of copulation. However, the function of the spermathecal eminence and the relationships between the spermathecal gland ducts, the accessory gland ducts and the atrium have remained obscure. Christophers (1960), and Curtin and Jones (1961) did not describe this region in detail. The presence of the thin-walled spermathecal vestibule connecting the ducts with the atrium is described for the first time. The dorsal plate, described by Curtin and Jones (1961), was not observed and probably represents the displaced genital lips of the female.

The peculiar ducted glands surrounding the base of the spermathecae and occurring in clusters along the spermathecal ducts are analogous in structure to the cells of the female accessory gland. Each cell contains a minute, flask-shaped, cuticular duct within its cytoplasm. Vacuoles, presumably secretory in nature, are contiguous with the ends of these ducts, and the nuclei and granular elements of the cell's cytoplasm are displaced to the opposite end of the cell. It is suggested, therefore, that each of these cell types is secretory. The spermathecal glands secrete into the spermathecae through the ducted pores around their base. The glands surrounding the spermathecal ducts appear to secrete into the ducts through their own minute ducts which presumably pierce the substance of the spermathecal ducts. The spermathecal ducts of *Anopheles gambiae* are pierced by the ducts of similar cells (Giglioli, 1963). Similarly, the female accessory gland apparently secretes directly into the spermathecal vestibule. Although the functions of these glands are unknown, they may be presumed to have some role in insemination.

The sensory stimuli that control copulation are apparently multiple. The sound of the female's wings (Roth, 1948), indeed, attracts males and induces them to perform certain pre-copulatory behavior patterns. Once the male has been primed by the sound of her wings, tarsal contact appears to induce further copulatory behavior. However, aedeagal eversion appears to be stimulated exclusively through contact of the male's terminalia with those of the female. It is suggested that the prominent, recurved sensillae on the basal segment of the clasper may receive such a stimulus from the post-genital plate of the female. Rees and Onishi (1951) observed that mechanical stimulation of these hairs caused movement in the aedeagus of *Culiseta inornata* males interrupted while copulating. Copulation of restrained *Aedes* pairs can be induced by placing the terminalia in contact (McDaniel and Horsfall, 1957). Thus, various stimuli mediated through the terminalia, as well as through the tarsi and antennae, control copulation and sperm transfer.

SUMMARY

1. The aedeagus of the *Aedes aegypti* male is placed superficially within the genital orifice of the female during copulation and is not a deep intromittent organ. When everted, it rotates through more than 90 degrees and its lobes spread, revealing the genital pore in its base. The paraprocts' function is to distend the genital lips of the female and to elevate the anal cone of the male. During coitus, the sleeve-like atrial membrane of the female is everted, revealing the genital opening at its apex. Sexual union is accomplishd through the junction of the everted aedeagus and the atrial membrane. The anal cone, the aedeagal pouch and the paraprocts assist in the formation of a firm line of union. The mechanics of eversion of the aedeagus and atrial membrane and the details of their juxtaposition are analyzed.

2. It is suggested that contractions of the male accessory glands provide the current in which the sperm are carried to the copulatory bursa of the female, and that the sperm subsequently transfer to the spermathecae.

3. The anatomy of the genital atrium of the *A. aegypti* female is described. The valves of the lower atrium seal the oviduct of the non-ovipositing female. The dorsal valve is everted during copulation and its spinous outer surface engages the

spine of the aedeagus. The spermathecal ducts and accessory gland duct communicate with the atrium through a common chamber.

4. Males receive complex mating stimuli from the female and require auditory as well as other kinds of stimuli before sperm transfer is accomplished.

LITERATURE CITED

Brelje, R. von der, 1924. Die Anhangsorgane des weiblichen Geschlechtsganges der Stechmucken (Culicidae). *Zool. Anz.*, **61**: 73–80.

Burcham, E. G., 1957. Some characteristics and relations of mating and oviposition of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae). Thesis, Ohio State University.

Christophers, S. R., 1960. *Aedes aegypti* (L.). The yellow fever mosquito. Cambridge Univ. Press, London.

Coher, E. I., 1948. A study of the female genitalia of Culicidae: with particular reference to characters of generic value. *Ent. Amer.*, **28**: 75–112.

Curtin, T. J., and J. C. Jones, 1961. The mechanism of ovulation and oviposition in *Aedes aegypti*. *Ann. Ent. Soc. Amer.*, **54**: 298–313.

Gigliol, M. E. C., 1963. The female reproductive system of *Anopheles gambiae melas*. *Riv. Malariol.*, **2**: 149–176.

Hertig, M., 1949. The genital filaments of *Phlebotomus* during copulation (Diptera, Psychodidae). *Proc. Ent. Soc. Wash.*, **51**: 286–288.

Hodapp, C. J., and J. C. Jones, 1961. The anatomy of the adult male reproductive system of *Aedes aegypti* (Linnaeus) (Diptera, Culicidae). *Ann. Ent. Soc. Amer.*, **54**: 832–844.

Komp, W. H. W., 1956. Copulation in crab-hole mosquitoes (Diptera, Culicidae). *Proc. Ent. Soc. Wash.*, **58**: 349–351.

Leahy, M. G., 1962. Barriers to hybridization between *Aedes aegypti* and *Aedes albopictus*. (Diptera: Culicidae). Thesis, Univ. Notre Dame.

Lum, P. T. M., 1961. The reproductive system of some Florida mosquitoes. 1. The male reproductive tract. *Ann. Ent. Soc. Amer.*, **54**: 397–401.

McDaniel, I. N., and W. R. Horsfall, 1957. Induced copulation of aedine mosquitoes. *Science*, **125**: 745.

Newcomer, E. H., 1953. A new cytological and histological fixing fluid. *Science*, **118**: 161.

Parks, J. J., 1955. An anatomical and histological study of the female reproductive system and follicular development in *Aedes aegypti* (L.). Thesis, Univ. of Minnesota.

Rees, D. M., and K. Onishi, 1951. Morphology of the terminalia and internal reproductive organs, and copulation in the mosquito, *Culiseta inornata* (Williston). *Proc. Ent. Soc. Wash.*, **53**: 233–246.

Roth, L. M., 1948. A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Amer. Midl. Nat.*, **40**: 265–352.

Russell, P. F., and B. N. Mohan, 1939. Insectary colonies of *Anopheles stephensi*. *J. Mal. Inst. India*, **2**: 433–437.

Snodgrass, R. E., 1963. A contribution toward an encyclopedia of insect anatomy. *Smithson. Misc. Coll.*, **146**, (*2*).

Wheeler, R. E., and J. C. Jones, 1960. The mechanics of copulation in *Aedes aegypti* (L.) mosquitoes. *Anat. Rec.*, **138**: 388.

EFFECTS OF MERCAPTOETHANOL ON THE FURROWING CAPACITY OF ARBACIA EGGS [1]

ARTHUR M. ZIMMERMAN [2]

Department of Zoology, University of Toronto, Toronto, Canada, and Marine Biological Laboratory, Woods Hole, Massachusetts

In recent years there have been several comprehensive reviews concerning the mechanisms of cell division (Gross, 1960; Mazia, 1961; Levine, 1963). However, the nature of the structural proteins believed to be involved in cytokinesis is still to be elucidated. Possibly, the answer to what mechanisms are responsible for cytokinesis could be clarified by an understanding of the role thiol groups play and their relationship to sol-gel reactions which accompany cytokinesis.

Thiol groups are known to play an important role in mitotic formation (Mazia, 1958; Mazia and Zimmerman, 1958). Inhibiting the thiol groups of cleaving cells with sulfhydryl inhibitors (salyrgan and p-chloromercuribenzoate) was found to block cytokinesis (Zimmerman *et al.,* 1957). Furthermore, the inhibition of the thiol groups is related to a weakening of the cortical plasmagel of the egg. The fluctuation in the —SH content of contractile thread models prepared from sea urchin eggs has also been studied with respect to elucidating the mechanisms of division (Sakai, 1962a, 1962b).

Experiments employing mercaptoethanol, a substance having a readily available source of thiol groups, have demonstrated that gelated structures within the cell, such as the mitotic apparatus (Mazia and Zimmerman, 1958), and the plasmagel of the amoeba (unpublished data), are markedly altered by the addition of mercaptoethanol. Since it has been proposed that the gelation reactions in the plasmagel are responsible for cytokinesis, mercaptoethanol, presumably by interfering with sol-gel reactions, should alter the structural characteristics of the plasmagel and thus affect cell division.

One tool that has been found to be useful in evaluating the effects of chemical agents on cell division is hydrostatic pressure. High pressure tends to solate the cortical plasmagel of cleaving eggs. Earlier studies (*cf.* Marsland, 1956) have shown that the structural characteristics of the cortical plasmagel are directly related to the cleavage capacity of the cell. Thus, the application of hydrostatic pressure blocks cell division. Since chemical agents may modify the structural gel components and consequently the cleavage capacity of the cell, these effects may be quantitated by measuring the pressure values essential to block division.

The present study was designed to investigate the effects of mercaptoethanol on the cleavage capacity of cleaving marine eggs. In addition, in order to establish

---

[1] The work was supported in part by Grant GM 07157-04, 05 from the Division of General Medical Sciences, United States Public Health Service.

[2] Part of this work was conducted while the investigator was affiliated with the Department of Pharmacology, Downstate Medical Center, State University of New York.

what effects thiol groups have on gelation reactions in the cell, pressure-centrifuge experiments were conducted on the cortical plasmagel of fertilized eggs.

MATERIALS AND METHODS

Living material. Eggs from *Arbacia punctulata* were obtained by means of 0.5-ml. intracoelomic injection of 0.53 *M* KCl. The shed eggs were washed three times by decantation with filtered sea water at 20° C. The sperm were obtained from excised testes and stored as "dry sperm" at 4° C.

Pressure apparatus. The microscope pressure chamber, patterened after one designed by Marsland (1950), with certain modifications, permits cells to be observed at magnification up to 600 times while being subjected to pressure treatment. Pressure was built up by means of an Aminco pressure pump at the rate of 5000 lbs./in.^{2}/second. The pressure was released almost instantaneously by means of a needle valve.

The centrifugal studies were conducted with a pressure-centrifuge head similar to one designed by Brown (1934). This pressure chamber contains two compartments, a control compartment and a pressure compartment. Thus, the experimental and control eggs can be simultaneously centrifuged. Centrifugal forces as high as 33,000 *g* were attained within a few seconds by means of a modified Dumore motor with an appropriate pulley system.

The microscope-pressure chamber and pressure centrifuge are mounted in a specially designed temperature chamber. The temperature control housing permits the temperature to be maintained at any point between −5° and 60° C. with a maximum internal variation of ±0.3° C.

Immersion procedure. At appropriate times (20–40 minutes) after insemination, the fertilized eggs were placed into a solution of mercaptoethanol at the desired concentration 0.01–0.075 *M*. After a 20-minute incubation in mercaptoethanol the eggs were subjected to centrifugal treatment. Centrifugal displacement of pigment vacuoles was established at a magnification of 440 ×.

In establishing the cleavage capacity of fertilized eggs, the cells were immersed in the mercaptoethanol 20 minutes prior to the expected time of furrowing. Subsequently, the cells were placed in the microscope-pressure chamber, and at the time of incipient furrows they were subjected to the desired pressure.

Chemicals. The 2-mercaptoethanol ($HSCH_2CH_2OH$) was obtained from Eastman Organic Chemicals, Rochester, New York. Fresh solutions of mercaptoethanol in sea water were prepared daily and equilibrated at the desired temperature prior to use.

RESULTS

Preliminary observations. In general, the data summarized in Table I are in good agreement with the earlier observations of Mazia and Zimmerman (1958). It appears, however, that the *Arbacia* eggs are slightly less sensitive to mercaptoethanol than the eggs of *Strongylocentrotus purpuratus.* When fertilized *Arbacia* eggs are placed into a blocking concentration of mercaptoethanol (0.075 *M*) prior to metaphase, the subsequent division is blocked. However, if the cells are placed into the same concentration of mercaptoethanol after metaphase, the cells divide, but do not progress past the two-cell stage. A decrease in the concentration of mercaptoethanol permits the cells to develop to more advanced stages, but they are subsequently blocked. When the cells are placed in 0.025 *M* mercaptoethanol, 20 minutes after insemination, the first division is delayed 2–9 minutes, and development is blocked at the two-cell stage. However, at lower concentrations, 0.01 *M*, the cells develop at a rate very close to that of the controls; occasionally, the first division is delayed up to four minutes, but in the majority of the treated cells, there is no delay. At 0.01 *M*, the embryos proceed to the blastula stage at a normal rate.

TABLE I

The effects of various concentrations of mercaptoethanol on developing Arbacia punctulata

Eggs immersed in mercaptoethanol 20 minutes after insemination	Stage of development							
Mercaptoethanol concentration	57 min. after insemination		75 min. after insemination		95 min. after insemination		20 hours after insemination	
	% cleaved	Stage	% cleaved	Stage	% cleaved	Stage	% cleaved	Stage
0.075 *M*	0	No division	0	No division	0	No division	0	No division
0.025 *M*	7	2-cell	98	2-cell	98	95% 2-cell 3% 4-cell	95	80% 2-cell 15% 3–4-cell
0.010 *M*	60	2-cell	92	2-cell	97	4-cell	97	16–32-cell
Control	55	2-cell	96	2-cell	96	4-cell	96	Free moving gastrulae

Pressure-centrifuge studies. Pressure-centrifuge studies may be used as a means of studying the physical state of the structural elements concerned with division. Previous studies have demonstrated that the cortical plasmagel of fertilized eggs becomes rigid following fertilization (Marsland, 1956), and the relative centrifugal force required to displace the echinochrome pigment vacuoles in the cortex of the egg can be taken as an indication of gel strength. It is possible, therefore, to use these physical characteristics to ascertain the effects of mercaptoethanol on the plasmagel of dividing eggs.

Two concentrations of mercaptoethanol were employed in these centrifugal studies, 0.075 *M* and 0.01 *M*. The fertilized eggs were immersed in the mercaptoethanol 20 minutes after insemination for a duration of 20 minutes, at which time they were subjected to centrifugal treatment. The experimental eggs were treated with pressure, whereas the control eggs were centrifuged at atmospheric pressure. In each test, the centrifugal time (at 33,000 *g*) required to produce a standard displacement of the pigment vacuoles lying in the cortical region was taken as an index of the relative strength of the gel. The standard displacement utilized was the removal of all but 3–6 of the pigment vacuoles from cortical cytoplasm in the hyaline region of the centrifuged eggs observed at a magnification of 440 ×.

Previous studies have shown that this end-point is reproducible and may be used as an index of plasmagel strength (Zimmerman *et al.*, 1957; Marsland and Zimmerman, 1963).

As shown in Figure 1, at each of the four pressure levels studied, the gel strength of the mercaptoethanol-treated eggs is consistently lower than the control eggs. The eggs immersed in the blocking concentration of mercaptoethanol, 0.075 *M*, exhibited a structural strength 22–24% lower than that found for the non-treated controls. At the lower mercaptoethanol concentration, namely 0.01 *M*, the division was not blocked and the gel strength curve was parallel to the curve for the blocking concentration and to the control curve, lying intermediate between the two.

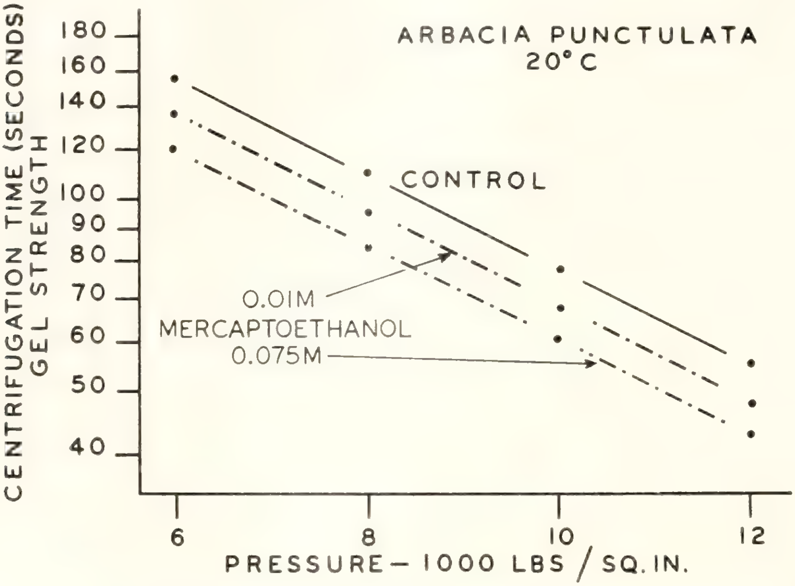


FIGURE 1. The effects of 0.01 *M* and 0.075 *M* mercaptoethanol on the gelational state of the cortical cytoplasm of fertilized *Arbacia* eggs. The gel strength is plotted as a function of pressure, at 20° C. The standard centrifugal force in these experiments was 33,000 *g*.

Each of the points plotted represents an average of 8–10 determinations. Different batches of eggs exhibit some variation in gel strength. However, in order to minimize variation, preliminary analysis permitted the selection of eggs which exhibited end-points at centrifugal times of 105–120 minutes at a pressure of 8000 lbs./in.^{2}. In all cases the slope of the curve did not vary. In those batches of eggs which exhibited high gel-strength, the slope was above that for the average. The occasional batch which exhibited lower gel-strength showed a slope slightly below the average. However, by preliminary selection of batches of eggs, variation remained ±5%. Since gel characteristics of eggs may change with increased time, we did not conduct more than ten determinations on a given batch of eggs.

Action of mercaptoethanol on cleavage capacity. Previously it was reported (Marsland, 1956; Zimmerman *et al.*, 1957) that the minimum pressure necessary to block cytokinesis may be used as an index of measuring the "furrowing or cleavage capacity" in dividing eggs. The cleavage potential reflects the ability of the cell to divide and may be compared in normal and treated cells. Furthermore, it has been shown that the furrowing capacity is a function of the gelation state of the cortical plasmagel. Thus, it was considered essential to ascertain the effect of mercaptoethanol on furrowing capacity of these eggs.

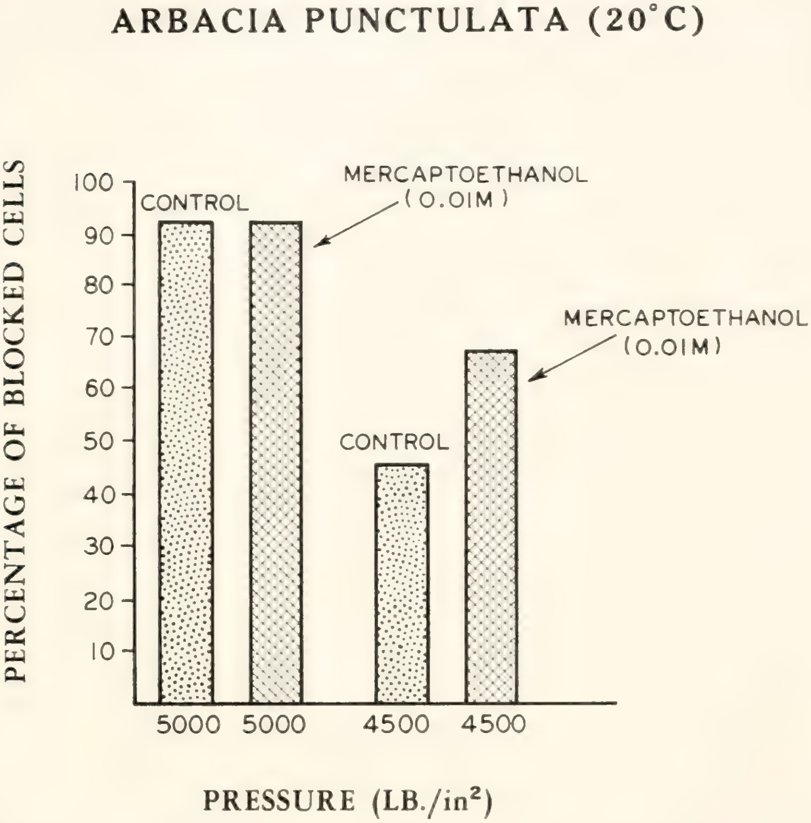


FIGURE 2. The effects of 0.01 *M* mercaptoethanol on the cleavage capacity of furrowing *Arbacia* eggs. In each experiment the percentage of blocked cells was determined after a standard compression period of 30 minutes.

The preliminary studies of varying concentrations of mercaptoethanol indicated that a concentration of 0.01 *M* has a minimal effect on first division. Therefore, this concentration was chosen for analysis of the effects of mercaptoethanol on the cleavage capacity.

The fertilized cells at 20° C. were immersed into 0.01 *M* mercaptoethanol, 20 minutes prior to the expected time of division, and subjected to a pressure of 4500 lbs./in.^{2}. After a 30-minute duration of pressure, the percentage of blocked cells was determined. In general, at this subcritical blocking pressure, 70% of the cells treated with mercaptoethanol were blocked. However, at the same pressure level [illegible] mercaptoethanol-treated eggs were studied. [illegible] pressure to 5000 lbs./in.² [illegible] at both the control and the mercaptoethanol-treated eggs, and no differential pressure effect could be established between the treated and control eggs at the higher pressure level (see Fig. 2).

DISCUSSION

[illegible] these studies demonstrated that [illegible] of the sea urchin egg is a [illegible] structure. In addition, the investigation shows that chemical substances which tend to [illegible] within the cell also alter the structural characteristics of the cleavage furrow. Furthermore, it was established that the structural changes [illegible] by these compounds [illegible] in the capacity of the cell to furrow.

Earlier studies (Mazia, 1958; Mazia and Zimmerman, 1958) have [illegible] mercaptoethanol [illegible] of mitotic structures. [illegible] of cell division with mercaptoethanol [illegible] the duplication of [illegible] (Mazia et al., 1961). Although [illegible] in the presence of mercaptoethanol, DNA synthesis is [illegible] (Bucher and Mazia, 1960).

The present [illegible] mercaptoethanol (0.075 M) [illegible] the structural [illegible] of the [illegible]. In order to [illegible] the effect of mercaptoethanol on the [illegible] concentrations of mercaptoethanol (0.05 M) were employed which did not block mitotic function. [illegible] of the "cleavage potential" [illegible] in the presence of [illegible] mercaptoethanol. In [illegible] the "cleavage capacity" and the "cleavage [illegible]" were [illegible] in the presence of mercaptoethanol. From the earlier work [illegible] in this laboratory and that of Marsland [illegible] the cleavage [illegible] of the cell [illegible] to be [illegible] surrounding the cell.

When [illegible] equilibrium within a cellular system is [illegible], the structural as well as functional aspects of cellular activity are [illegible]. There is strong evidence [illegible] SH groups [illegible] that play an [illegible] role in the [illegible]. The [illegible] has been shown to be a function of [illegible]. The structural [illegible] of the [illegible] can be changed when the [illegible] (Marsland and Zimmerman, 1963; Zimmerman, 1963, [illegible] 1960, and [illegible] et al., 1963). Therefore it has been observed (Zimmerman et al., 1963) that [illegible] "cleavage capacity" and the [illegible]. [illegible] tions associated with pseudopodial stability and amoeboid movement (unpublished data). *Amoeba proteus* treated with mercaptoethanol exhibits a marked lowering of pseudopodial stability, accompanied by a weakening of the plasmagel (ectoplasm). Thus, the lowered "cleavage potential" and the decreased plasmagel rigidity in the *Arbacia* egg following mercaptoethanol treatment are to be expected if the plasmagel structure of the cell, just prior to division, is in a dynamic state shifting from thiol to disulfide bonding structures. This indeed appears to be true. Sakai (1963) has shown that the thiol content of isolated sea urchin cortices reaches a maximal —SH content at metaphase and diminishes as the cell prepares for division. By stabilizing the —SH content of the cortex of the egg with etherized sea water, cytokinesis is blocked. Following removal of ether-blocked eggs to normal sea water, the —SH content of the bound protein within the cortex diminishes as cytokinesis proceeds. Sakai (1962a, 1962b) has reported changes in contractility of thread models prepared from fertilized sea urchin eggs which relate to developmental stages. The changes in contractility of these models are accompanied by changes in the —SH content of the KCl-soluble egg proteins.

SUMMARY

1. The fertilized eggs of *Arbacia punctulata* were immersed into various concentrations of mercaptoethanol, and the structural state of the cortical cytoplasm, as well as the "cleavage potential" of the cells, were measured.

2. Pressure-centrifuge measurements of the structural state of the cortical cytoplasm were made at various pressures (6000–12,000 lbs./in.^{2}) at 20° C., employing a centrifugal force of 33,000 g. A blocking concentration of mercaptoethanol, 0.075 M, yielded a value for the strength of the cortical gel which was 22–24% lower than that found in the non-treated controls. At a lower mercaptoethanol concentration, 0.01 M, division was not blocked and the gel strength curve was parallel to the curve for the blocking concentration and the control curve, but lying intermediate between the two.

3. The decrease in the gel strength was shown to be related to a decrease in the "cleavage potential." A pressure of 4500 lbs./in.^{2} applied at the time of furrowing will, in general, block about 50% of the cells from cleaving. When the eggs were pretreated with 0.01 M mercaptoethanol 20 minutes prior to division, there was a 24% lowering in the number of cells which completed division under pressure treatment, as compared to the non-treated pressurized controls.

4. In general, the data support the hypothesis that interference with the $SH \rightleftharpoons S-S$ interaction in protoplasmic gel system is similar in both the mitotic gel system and the cortical gel system, and any interference with the delicate balance may markedly disrupt mitosis and cytokinesis.

LITERATURE CITED

Brown, D. E. S., 1934. The pressure coefficient of "viscosity" in the eggs of *Arbacia punctulata*. *J. Cell. Comp. Physiol.*, **5**: 335–346.

Bucher, N. L. R., and D. Mazia, 1960. Desoxyribonucleic acid synthesis in dividing eggs of the sea urchin, *Strongylocentrotus purpuratus*. *J. Biophys. Biochem. Cytol.*, **7**: 651–655.

Gross, P., (ed.), 1960. Second conference on the mechanisms of cell division. *In: Ann. N. Y. Acad. Sci.*, **90**: 345–613.

Levine, L., (ed.), 1963. The Cell in Mitosis. Academic Press Inc., New York.

Madsen, N. B., and C. F. Cori, 1956. The interaction of muscle phosphorylase with p-chloromercuribenzoate. *J. Biol. Chem.*, **223**: 1055–1065.

Marsland, D., 1950. The mechanism of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. Comp. Physiol.*, **36**: 205–227.

Marsland, D., 1956. Protoplasmic contractility in relation to gel structure: temperature-pressure experiments on cytokinesis and amoeboid movement. *Intern. Rev. Cytol.*, **5**: 199–227.

Marsland, D., and A. M. Zimmerman, 1963. Cell division: differential effects of heavy water upon the mechanisms of cytokinesis and karyokinesis in the eggs of *Arbacia punctulata*. *Exp. Cell Res.*, **30**: 23–35.

Mazia, D., 1958. SH compounds in mitosis. I. The action of mercaptoethanol on the eggs of the sand dollar *Dendraster excentricus*. *Exp. Cell Res.*, **14**: 486–494.

Mazia, D., 1961. Mitosis and the physiology of cell division. *In:* The cell, Brachet, J. and A. E. Mirsky, Eds., Academic Press, Inc., New York. Vol. **3**: 77–412.

Mazia, D., and A. M. Zimmerman, 1958. SH compounds in mitosis. *Exp. Cell Res.*, **15**: 138–153.

Mazia, D., P. J. Harris and T. Bibring, 1960. The multiplicity of the mitotic center and the time-course of their duplication and separation. *J. Biophys. Biochem. Cytol.*, **7**: 1–20.

Sakai, H., 1962a. Studies on sulfhydryl groups during cell division of sea urchin egg. IV. Contractile properties of the thread model of KCl-soluble protein from the sea urchin egg. *J. Gen. Physiol.*, **45**: 411–425.

Sakai, H., 1962b. Studies on sulfhydryl groups during cell division of sea urchin egg. V. Change in contractility of the thread model in relation to cell division. *J. Gen. Physiol.*, **45**: 427–438.

Sakai, H., 1963. Studies on sulfhydryl groups during cell division of sea urchin egg. VI. Behavior of -SH groups of cortices of eggs treated with ether-sea water. *J. Gen. Physiol.*, **32**: 391–393.

White, J. I., H. B. Bensusan, S. Himmelfarb, B. E. Blankenhorn and W. R. Amberson, 1957. △ Protein, a new fibrous protein of skeletal muscle: properties. *Amer. J. Physiol.*, **188**: 212–218.

Zimmerman, A. M., 1960. Physico-chemical analysis of the isolated mitotic apparatus. *Exp. Cell Res.*, **20**: 529–547.

Zimmerman, A. M., 1963. Chemical aspects of the isolated mitotic apparatus. *In:* The Cell in Mitosis, Levine, L., Ed. Academic Press, Inc., New York. Pp. 159–184.

Zimmerman, A. M., J. V. Landau and D. Marsland, 1957. Cell division: a pressure-temperature analysis of the effects of sulfhydryl reagents on the cortical plasmagel structure and furrowing strength of dividing eggs (*Arbacia* and *Chaetopterus*). *J. Cell. Comp. Physiol.*, **49**: 395–435.

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1964

ABSTRACTS OF SEMINAR PAPERS

July 7, 1964

Differential sorting of shells in the swash zone. J. Stewart Nagle.

Experiments were performed under varied wave conditions on sandy beaches of slopes of less than five degrees in Falmouth and Woods Hole, Massachusetts, and Sapelo Island, Georgia, to determine a possible cause of differential separation of right and left valves of pelecypod shells on beaches.

It was found that the left-right phenomenon is responsible for swash zone separation of most pelecypod shells in which the right and left valves are mirror images.

To produce this separation, waves must approach the shore askant. Water in the swash zone moves onshore obliquely, describes an arcuate path at maximum flood, and returns to sea at approximately right angles to the shoreline.

Generally, when two separate, opposite valves are moved into the swash zone by large waves, they are translated and rotated, but shortly are oriented so that the beaks are pointed seaward by the outgoing water. As the next wave approaches slantwise, the beak of one valve faces into the wave while the beak of the other faces away from it. This wave moves the valve whose beak is facing the oncoming waves; and successive waves continue the translation-rotation-orientation process until the shell is either removed from the swash zone or drifted to centers of beach cusps. The opposite valve, however, remains in place, so is deposited near the location where it was originally brought into the swash zone.

When large numbers of valves of all sizes are present, differential separation is most complete with the largest valves; but is less so with smaller valves, because they are easily moved in any orientation, and are deposited more by chance than through orientational stability. Rate of separation is faster with increasing obliqueness of wave approach, as the beaks of one set of valves face more directly into the waves approaching at greater angles, so are more easily moved.

Supported in part by Grant GB-561 from the National Science Foundation.

Comparative deviations in motility of developing sea urchins induced by irradiation. (With motion picture). Carl Caskey Speidel and Ralph Holt Cheney.

Comprehensive observations were made of abnormal motility exhibited by developing *Arbacia* following 2537 Å ultraviolet or x-ray treatment of sperm alone, eggs alone, or zygotes, the zygotes being irradiated at early non-motile stages from fertilization to hatching and at later motile stages from blastula to young pluteus. Significant differences and resemblances in motility abnormalities were noted. Analysis of cinephotomicrographic records revealed that such differences and resemblances were correlated with the type and strength of irradiation, the three kinds of material irradiated, and, for zygotes, the stage of development when irradiated.

X-ray irradiation is ionizing, deeply penetrating, and non-selective. UV irradiation is non-ionizing, poorly penetrating, and very selective. *Both types probably alter DNA,* thus inducing developmental abnormalities.

Following unilateral UV irradiation of eggs alone, or of *non-motile* zygotes, interesting progeny resulted. Many individuals exhibited either abortive or complete autotomy accompanied by conspicuous deviations in motility. Such autotomy and such deviations were markedly different from those induced by unilateral UV irradiation of *motile* zygotes. The difference was correlated with one-sided exposure in the first case and exposure from all sides in the second. X-ray (gamma) irradiation of eggs alone, or non-motile zygotes, induced motility deviations unlike those that followed similar UV irradiation.

UV irradiation of sperm alone induced developmental abnormalities essentially like those induced by x-irradiation of sperm alone. The small size and movement of the sperm cell rendered its DNA content vulnerable to alteration by poorly penetrating UV rays, as well as deeply penetrating x-rays. A frequent early site for sloughing of injured cells in developing embryos was at the blastopore; a later site was near the apex of the young pluteus.

Certain differences in motility deviations, observed in embryos arising from irradiated sperm alone, compared with those from irradiated eggs or zygotes, were correlated with differences in the cytoplasmic complex irradiated.

Supported by Grants GM 04326-07 and RH 00325-01 to C.C.S. from the U.S.P.H.S.

JULY 14, 1964

The sites of nuclear RNA synthesis during amphibian embryogenesis. SHUICHI KARASAKI.

The site of H^3-uridine incorporation and the fate of labeled RNA were studied with electron microscopic autoradiography and cytochemical techniques. Isolated tissues from the embryos of *Triturus* were treated in H^3-uridine (50 μC./ml.) for three hours and cultured in cold solution for various periods before fixation with OsO_4 and embedding in Epon. Glycidaldehyde fixation and glycolmethacrylate embedding, followed by digestion with DNase, RNase and pepsin have been also employed. Previous to the gastrula stage, most of the H^3-uridine is incorporated into the DNA of the nuclear chromatin fibrils. At the early gastrula stage, labeled RNA begins to appear in the primary nucleoli, which originate as small dense fibrous bodies within the chromatin material. At the tail-bud stage and later, most of the H^3-uridine is incorporated into nuclear RNA with the nucleolus consistently showing more label per unit area than the rest of the nucleus. During the same stages, labeled RNA is first localized in the central region of the nucleolus and on the periphery of the nuclear chromatin strands. After longer culture in nonradioactive medium, labeled materials begin to appear in the peripheral region of the nucleolus and in the interchromatin spaces. Further incubation in cold solution gives labeling in the cytoplasm. The nucleoli enlarge during these developmental stages by the acquisition of ribosome-like particles, which first appear centrally in the nucleoli and then form a layer around the dense fibrous component. Simultaneously, large particles (300–500 Å) appear in the nuclear chromatin and fill the interchromatin spaces by the tail-bud stage. In many nuclei, particularly those exhibiting the greatest incorporation, the chromatin fibrils are dispersed. By enzyme digestion and uranyl staining procedures, the presence of the submicroscopic chromatin fibrils was demonstrated in the internal fibrous portion of the nucleolus. These chromatin fibrils may be the initial site of nucleolar RNA synthesis.

Research supported by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

Density gradient media for mammalian spermatozoa. RICHARD A. BEATTY.

Motile rabbit spermatozoa have been centrifuged to equilibrium in density gradients at 20 C. First, *BM saline* was developed (pH 7.6); 1.00 strength BM (osmotic pressure = 1% NaCl) comprised glucose 3.362 g., NaCl 0.252 g., KH_2PO_4 0.0353 g., Na_2HPO_4 0.252 g., deionized water to make 100 ml. Motility and fertility were optimal at 0.93 strength. *Colloidal silica* dialyzed in closed cellophane bags against 0.93 BM for $\geqq$ 2 days covered the necessary specific gravity range, the spermatozoa being motile though irreversibly infertile. After centrifugation to near-equilibrium (3000 r.p.m. for 30 minutes), 95% of spermatozoa in silica gradients were in the specific gravity range 1.09–1.18, and 50% in the range 1.11–1.14; mean specific gravity was 1.132. Bands of material were visible at equilibrium, with apparent centers at specific gravity 1.045 (kinetoplasmic globules?), 1.09 (band perhaps double or treble, spermatozoa bearing globules), 1.125 (probably double, spermatozoa without globules), 1.145 (spermatozoa without globules), and *ca.* 1.18 (small band with some isolated heads). *Crystallized bovine plasma albumen* in 0.9% or 0.45% NaCl gave excellent fertility but was too viscous above specific gravity 1.11. *Dextran* ("Ficoll"), made up in BM strengths giving optimal motility and fertility, allowed spermatozoa to return fertile up to specific gravity *ca.* 1.13. A few mixtures are as follows, the five figures in each set being respectively dextran g./100 g. solution; BM strength; specific gravity 20°/20° C.; osmotic pressure as NaCl %-age w/v equivalent; pH, 27.382, 0.41, 1.110, 1.2, 7.2; 32.687, 0.25, 1.130, 1.5, 7.1; 37.893, 0.069, 1.150, 1.9, 6.7. Preliminary experiments showed no differential effect on sex ratio of offspring after inseminating rabbit spermatozoa separated on dextran gradients into fractions of specific gravity either $\geqq 1.13$ or $\leqq 1.13$.

I am grateful for donations of Syton-2X (Monsanto Chemicals) and Ludox-HS (DuPont de Nemours and Co., Inc.)

JULY 21, 1964

Analysis of the biological excitable membrane by means of voltage-current-time characteristics. SHOJI HIGASHINO.

It was made clear that the voltage-current characteristic curve of the excitable membrane obtained by the voltage clamp methods developed by Hodgkin and Huxley changed as a function of time.

Some types of behavior of the biological excitable membrane, such as the initiation of action potential, the threshold, the period of relative refractoriness, the oscillation, and the after-potential, etc., which have hitherto been interpreted from the ion hypothesis, were analyzed from the voltage-current-time characteristics of the membrane, and from the theory and the experimental data of the tunnel diode which has stable and unstable points and also negative resistance and inductance components.

It was made clear that, when the electron conduction in the tunnel diode and the ion conduction in the biological excitable membrane, which are two quite different physical phenomena, are compared, both phenomena have the same nature from a viewpoint as non-linear oscillatory systems.

Further studies on incorporation of H^3 thymidine in Arbacia eggs under hydrostatic pressure. ARTHUR M. ZIMMERMAN AND LESTER SILBERMAN.

Fertilized eggs of *Arbacia punctulata* were placed into H^3 thymidine-sea water solution (1–2 μC./ml.) and subjected to pressures of 5000–15,000 lbs./in.^{2} at 20° C. for durations of 10–60 minutes. Following pressure treatment the eggs were placed into fixative. The paraffin-imbedded material was sectioned and subjected to autoradiographic procedures. The incorporation of tritiated thymidine was employed as an index of DNA synthesis. The uptake of H^3 thymidine was compared in the pressurized eggs and non-pressurized controls.

Eggs which were placed in H^3 thymidine (at 20° C.), but not pressurized showed incorporation of the isotope only in the interval from 15 to 30 minutes after insemination, corresponding to the time from syngamy to early streak. There was no evidence of H^3 thymidine incorporation into the pronuclei during the presyngamy period. However, eggs in which pronuclear fusion had been inhibited by a pressure of 5000 lbs./in.^{2} for 30–60 minutes showed incorporation in both male and female pronuclei.

It was found that 5000 lbs./in.^{2} did not block DNA synthesis, even in the face of cessation of morphological mitotic activity. However, pressures of 7500, 10,000 and 15,000 lbs./in.^{2} blocked DNA synthesis.

The results are discussed in terms of the effects of pressure on development and the independence of the DNA synthesis cycle and cell division.

The work was supported by grant GM 07157-05 from the Division of General Medical Sciences, U.S.P.H.S.

High pressure reversal of the anti-mitotic effects of heavy water (D_2O). DOUGLAS MARSLAND.

As previously reported, replacement of 70% or more of the H_2O content of sea water by D_2O immediately stops mitotic activity in cleaving sea urchin eggs, regardless of the stage of mitosis at which the experimental treatment is initiated. The mitotic apparatus remains "frozen" at *status quo,* whether in prophase, metaphase or anaphase, unless and until the eggs are returned to normal sea water within approximately one hour, before deterioration occurs.

In the present experiments, immersion of the eggs (*Strongylocentrotus purpuratus*) in the deuterated media (70%, 80% and 90% D_2O-sea water) was initiated at very early prophase and a sample of each batch of deuterated eggs was subjected to compression six minutes later. Without pressure, none of the eggs succeeded in reaching telophase, as judged by the appearance of cleavage furrows, whereas more than 98%, at optimum pressure, displayed definitive furrowing activity and about 60% succeeded in reaching the two-cell stage. The optimum pressure, however, increased as the deuteration increased, being 4000, 4500 and 5000 lbs./in.^{2}, respectively, for the 70%, 80% and 90% degrees of deuteration. Moreover, the percentage of development to morphologically normal swimming blastulae, in specimens restored to H_2O-sea water after one hour of experimental treatment tended to parallel the percentage of successful cleavage.

Similar results have also been obtained in reference to first cleavage in the eggs of the sand dollar, *Echinarachnius parma* and to the maturation divisions of the öocytes of the starfish, *Asterias forbesi.*

A reasonable interpretation of these results would be that pressure, by virtue of its widely recognized solational effect, tends to counteract the hypergelational "freezing" of the spindle-aster complex.

Work supported by grant series CA 00807 from the National Cancer Institute, U.S.P.H.S.

JULY 28, 1964

Production of inflammatory changes in the microcirculation by cationic proteins extracted from lysosomes. AARON JANOFF AND BENJAMIN W. ZWEIFACH.

The agents which mediate inflammatory changes in the microcirculation are not completely identified. Although certain vasoactive amines, indoles and polypeptides affect the permeability of small blood vessels to plasma proteins, none of these substances has been successfully implicated in leucocyte-sticking and emigration. Recent studies by Hurley, Spector, Moses and others suggest that the mediators responsible for the chronic influx of leucocytes during inflammation may be derived from the leucocytes themselves.

Lysosomal granules were isolated from polymorphonuclear (PMN) leucocytes from rabbit exudate and lysed by freezing-thawing. Application of this material to rat and rabbit mesentery produced sticking and emigration of leucocytes, stasis of blood flow, and petechial hemorrhage. The granule-free supernatant fraction of the homogenized leucocytes was inactive. Cationic proteins extracted from leucocyte granules by weak acid and precipitated by ethanol were then tested on homologous and autologous mesentery. A protein fraction, precipitated by 20% ethanol, duplicated the aforementioned inflammatory changes, whereas protein precipitated by 45% ethanol was inactive. The active protein was not pyrogenic, and exhibited no musculotropic effects when tested on isolated smooth muscle preparations. UV-absorption spectra and ribose assays showed that the fraction was essentially free of contamination with nucleic acid. Starch-gel electrophoresis in acid buffer separated the active protein into three components which migrated toward the cathode. The inflammatory protein fraction contained no acid-phosphatase, beta-glucuronidase, acid-ribonuclease, lysozyme, or catalase activity. The inactive protein fraction (45% ethanol) contained appreciable lysozyme and ribonuclease activity.

The foregoing data show that cationic proteins present in the lysosomes of rabbit exudate, PMN leucocytes can reproduce a cardinal feature of the inflammatory response, namely, adhesion and emigration of leucocytes in the microcirculation. These findings offer support for the role of leucocytes (especially the lysosomes of these cells) in the pathogenesis of inflammation.

Supported by U.S.P.H.S. grant HE 08192-01.

Condensation of the sperm nucleus and alignment of DNA molecules during spermiogenesis in Loligo pealii. HIDEMI SATO AND SHINYA INOUÉ.

The progressive change of nuclear structure during the course of sperm maturation was followed in living squid sperm with the phase-contrast and the rectified polarizing microscopes. Five distinct stages of spermiogenesis can be identified cytologically.

During spermiogenesis the telophasic nuclei of young spermatids, without going through interphase, change into "prophasic" nuclei showing many coils of chromonemata which are arranged more or less longitudinally in the sperm head (stages 1–2). The oriented chromonemata become more compact and short, also becoming more densely packed in the sperm head towards the end of maturation (stages 3–4). No resolvable structure exists in the mature sperm head (stage 5). The sperm nucleus gradually condenses laterally but does not change in length (*ca.* 7.8 μ) during the process of maturation. The final decrease in volume is four-fold (from 17 μ^3 to 4.3 μ^3). The birefringence (retardation) of the sperm nucleus changes from almost zero to thirty mμ during spermiogenesis. Since the width of the mature sperm is 1.4 μ, the coefficient of birefringence reaches 2×10^{-2}. This coefficient of birefringence which is negative in sign is characteristic of well oriented DNA gels. During maturation the osmotic response of the sperm head decreases gradually. In hypotonic solutions stage 1 (youngest spermatid) shows drastic increase of the nuclear volume, while stages 2, 3 and 4 show progressively less change. The mature sperm head shows no swelling, even in distilled water. No significant change of retardation is detected, however, when the sperm nuclei are swollen with hypotonic solution.

We may therefore conclude that the alignment of DNA molecules does not arise from the passive anisotropic compression of a DNA-protein gel but from a change in the intrinsic property of DNA-containing structure presumably undergoing a phase separation during sperm maturation.

Supported in part by grants from the National Science Foundation (GB-2060) and the National Cancer Institute, U.S.P.H.S. (CA 04552).

Cartilage in a marine polychaete, Eudistylia polymorpha. PHILIP PERSON.

In previous studies, we provided morphologic and chemical data establishing the presence of cartilage tissues in arthropods and molluscs, *e.g., Limulus, Busycon, Loligo,* etc. We wish now to report that we have found cartilage tissues in the marine polychaete, *Eudistylia.* Live specimens were obtained from Dr. Rimmon C. Fay, Pacific Bio Marine Co., Venice, California. Visual examination and dissections indicated the presence of cartilage-like material in the head and tentacular regions. Tissue specimens were removed and processed for hematoxylin and eosin staining of paraffin-embedded sections. Fresh-frozen sections were stained with toluidine blue. Freshly dissected tissues were carefully trimmed of extraneous tissues for analysis of mucopolysaccharide content (by Dr. Martin B. Mathews). Light-microscope examination of sections showed the presence of at least three distinct tissue types in different regions of the animal. Each consisted of cells suspended in a matrix, and possessing characteristic architecture of cartilage tissues. Metachromasia was seen in many areas of the toluidine blue-stained sections, both in the matrix and intracellularly. Preliminary chemical analyses by Dr. Mathews have indicated the presence of hexuronic acid, hexosamine and ester sulfate. A detailed report is in preparation.

AUGUST 4, 1964

Further studies on the cell division without mitotic apparatus in sea urchin eggs.
YUKIO HIRAMOTO.

In order to examine whether or not the presence of the mitotic apparatus is indispensable for cleavage, the following experiments were carried out in the eggs of the heart-urchin, *Clypeaster japonicus.* When a large quantity of paraffin oil was injected into the center of the egg shortly before the onset of cleavage, the mitotic apparatus was very much displaced and deformed by the injected oil drop. Cleavage started in such an egg in a virtually normal manner and the oil drop was constricted by the advancing furrow. When a large quantity nic sucrose solution or sea water was injected into the egg, the mitotic apparatus was pletely disintegrated by direct contact with these solutions. The injected sucrose solution mixed with the protoplasm fairly well, while a precipitation membrane was formed around injected sea water. Cleavage took place in both of the above cases. Furrowing took place in some eggs in which more than 50% of the endoplasm had been replaced with sea water before onset of cleavage, although the furrow regressed afterwards. From these experiments, it was concluded that cleavage may take place without the mitotic apparatus and that a difference in contractility between the furrow and the polar regions of the cell cortex is probably the immediate cause of cleavage.

GERL, its form and function in neurons of rat spinal ganglia. ALEX B. NOVIKOFF.

We have suggested that smooth endoplasmic reticulum (ER) in the Golgi zone of rat hepatocytes may form two kinds of lysosomes, dense bodies and autophagic vacuoles. In motor neurons of rat spinal cord, some images suggest accumulation of ferritin-like grains within smooth ER adjacent to Golgi saccules, and the formation of dense bodies from these regions of ER. This suggestion gains support from observations on spinal ganglion neurons. The smaller neurons in these ganglia possess a high nucleus-to-cytoplasm ratio and large, multiple nucleoli, and their multiple dictyosome-like Golgi elements lie in a perinuclear zone. Between the cell nucleus and the innermost of the three Golgi saccules there is a region of smooth ER with a complex, specialized structure in which localized accumulations of ferritin-like grains and continuities with "coated vesicles" are common. The images suggest that both dense bodies (lysosomes) and "coated vesicles" arise from it. Its continuity with ribosome-studded ER has been observed but not with Golgi saccules; serial sections are now being studied. The innermost Golgi saccule appears fenestrated at its lateral edges and, like the specialized region of smooth ER, it shows acid phosphatase activity and nucleoside monophosphate activities at pH 5 and 7.

The specialized region of ER is referred to as GERL, to suggest that it is intimately related to the Golgi saccule (G), that it is part of the ER, and that it forms lysosomes (L). The observations raise the possibilities that intracellular digestion occurs in GERL; that some digestive products are moved into the cytoplasm through "coated vesicles" in a pinocytotic process; and that some undigested residues accumulate as ferritin-like grains and membranous arrays. Tracer experiments are needed to determine the extent to which products of metabolic (ribonucleoprotein, deoxyribonucleoprotein?) activities and exogenous materials gain access to GERL.

AUGUST 11, 1964

A cold precipitable protein in the dogfish lens. SEYMOUR ZIGMAN, JUDY MUNRO AND SIDNEY LERMAN.

The mammalian lens contains a protein fraction (C.P.P.) which precipitates at temperatures below 10° C. This phenomenon occurs in the young lens and in lens homogenates of any age group. Urea at 0.3 *M* concentration inhibits this cold precipitation effect. The dogfish lens has a urea concentration of 0.25 to 0.30 *M*, which prevents cold precipitation, but when the urea is dialyzed out, a cold-precipitable protein can be obtained.

This protein exhibits characteristics similar to the C.P.P. in the rat lens. It decreases in concentration as the animal ages, from a level of 17% of the total soluble protein in the young dogfish and 35% in the foetal rat lens to 5–7% in the older animals. In both species the C.P.P. fraction contains 1.5% (by weight) RNA. Ultracentrifugal analysis of the dogfish or rat lens C.P.P. at 20° C. (pH 8.0) reveals a single peak with a sedimentation constant of 4s. At 4° C. and pH 3.0 the single peak is replaced by a minor 3s and a major 13s peak for the dogfish and a 3s and 17s peak for the rat.

Gell filtration of purified dogfish lens C.P.P. on Sephadex G-100 columns (Tris buffer 0.025 *M*, pH 7.4) separates it into two peaks. The first peak contains two species of lens protein, similar in sedimentation rate to that of alpha and beta crystallin; the second peak contains a single fraction with a sedimentation rate of gamma crystallin.

These data indicate that the dogfish lens contains a cold-precipitable protein fraction similar in physical and chemical properties to that of mammalian lenses. Urea is normally present in the dogfish lens at a concentration sufficient to prevent the precipitation of this cold protein when the fish is in an environment below 10° C.

This work was supported by A.E.C., and O.N.R. grants (M.B.L.) and U.S.P.H.S. grant NB 3081, and by a Fight for Sight Fellowship, National Council to Prevent Blindness.

The reaction of sodium borohydride with rhodopsin and intermediates of its bleaching. DERIC BOWNDS AND GEORGE WALD.

The visual pigment rhodopsin is composed of a protein, opsin, to which the 11-*cis* isomer of retinal (vitamin A aldehyde, retinene) is attached in a Schiff-base linkage: retinal-CH=O + H_2N-opsin → retinal-CH=NH^+ − opsin + OH^-. On absorbing light, rhodopsin bleaches over a succession of transient intermediates, stable only at low temperatures: pre-lumirhodopsin, lumirhodopsin, metarhodopsin I, metarhodopsin II, hydrolyzing finally to free retinal and opsin. Sodium borohydride, though it has no effect on rhodopsin itself, reduces the carbon-nitrogen double bond of the Schiff-base linkage of certain intermediates when rhodopsin is bleached in its presence. This replaces the labile Schiff-base linkage by a stable secondary amine linkage (retinal-CH_2-NH-opsin), binding the retinal firmly to opsin at the site of chromophore attachment. We call this reduction product N-retinyl-opsin. Its absorption maximum is at 333 mμ, close to that of retinol (vitamin A, 328 mμ). The intermediate of rhodopsin that is most readily reduced by sodium borohydride appears to be metarhodopsin II (λ_{max} 380 mμ). Metarhodopsin I (λ_{max} 478 mμ) either is not reduced at all or reacts very slowly. The fact that metarhodopsin II is reduced by borohydride indicates that in this intermediate the Schiff base linkage is maintained. The low reactivity of rhodopsin and earlier intermediates may be caused by inaccessibility of the retinal-opsin linkage, or by the fact that in all these forms the Schiff-base nitrogen is protonated, the proton being lost in the transition, metarhodopsin I → metarhodopsin II.

Supported by N.I.H. Training Grant to the Biology Dept. of Harvard University and by N.S.F. and O.N.R. grants to G. W.

AUGUST 18, 1964

On the chromosone number of Arbacia punctulata. WALTER AUCLAIR.

The chromosomes of *Arbacia punctulata* eggs have been relatively inaccessible for study and experimentation because of their small size and the lack of available staining methods for whole eggs, necessitating the use of sectioning procedure. A method has been devised which takes advantage of the nuclear staining "artifacts" of the Gomori technique for acid phosphatase localization. The important criteria of the method are: (1) the use of a dehydrating fixative, 50% ethanol or 50% acetone; (2) placing the dehydrated specimens directly into a medium containing 0.05% lead nitrate and an organic phosphate, such as adenosine 5′-monophosphate (0.01 *M*), which is hydrolyzed very little by the cellular phosphatases; and (3) maintenance of this medium at a pH of 4.5 to 5.5 with 0.2 *M* Tris-maleate buffer. As with the Gomori technique the cells are placed in ammonium sulfide following incubation in the lead-organic phosphate solution. The mechanism of the staining reaction is apparently a selective surface adsorption of lead ions, reinforced by the organic phosphate.

The present study of chromosome squash preparations of both colchicine-treated eggs and anaphase spreads gives a consistent number of 44 chromosomes. This is in contrast to earlier studies, summarized by Harvey, of counts from 34 to 40. The metaphase chromosomes are 3–6 microns long and have a characteristic constriction, or kinetochore. Using these two morphological features it has been possible to pair the chromosomes and form a karyotoype. Preliminary work with another echinoid, *Echinarachnius parma,* indicates a chromosome number of 44 or 46, indicating the possibility that a number in this range might be characteristic of the echinoids.

Supported in part by U.S.P.H.S. grant CA 06439-02.

Re-evaluation of the late Cenozoic cirriped "Tamiosoma" Conrad. VICTOR A. ZULLO.

"*Tamiosoma gregaria*," an exceptionally large barnacle peculiar to the late Cenozoic of the Pacific Coast of North America, was first described by Conrad (1856) from the elongate cellular bases. He determined the form to be a rudistid pelecypod, but later recognized that "*T. gregaria*" was a *Balanus* that had developed a basis structurally identical to that of *B. laevis* Bruguière.

Pilsbry (1916) formally synonymized *Tamiosoma* with *Balanus s.s.* on the basis of new collections which contained compartmental plates. However, it was not until the opercular valves were described by Woodring (1940) that any attempt could be made to determine the affinities of this unique species. Woodring concluded that *B. gregarius* was related to the *B. concavus* Bronn group (a complex prominent in Tertiary Tethyan-type faunas).

Examination of specimens both from recorded and new localities has disclosed three previously unrecognized facts: (1) the tergum of *B. gregarius* has a prominent beaked apex, (2) not all individuals, even from the same locality, have specialized bases, and (3) fossils of this species occur in deposits ranging in age from early Miocene through Pleistocene in central, southern and Baja California.

Comparison of well preserved opercular valves with those of the extant Pacific Coast species *B. aquila* reveals no apparent differences. The only major distinction in the two species would appear to be a tendency for the formation of elongate cellular bases in the fossils. However, this unique basis has now been discovered in extant specimens of *B. aquila* taken by the Allan Hancock Foundation off Santa Rosa Island, California. It is probable that the prevalence of elongate bases in fossils is related to past environmental conditions (warm, shallow embayments with high sedimentation rates) which are not widespread along the Pacific Coast today.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

Preliminary quantitative study of small-scale environmental and faunal variability in Hadley Harbor. ROBERT H. PARKER, J. STEWART NAGLE, ANDREW L. DRISCOLL AND KAREN LUKAS.

A detailed study of benthos of Hadley Harbor Complex, Woods Hole has been in progress for 10 months to relate quantitatively faunal occurrence and diversity to small-scale variability of multiple ecologic parameters. Within the one-half square mile under investigation, 87 quantitative samples for faunal analyses were collected with a small Van Veen grab, and 3000 *in situ* physico-chemical measurements were taken. Grab samples were sieved through five Wentworth screens grading from 4 to 0.250-mm. mesh openings. Mollusks were identified to species and other organisms to major taxa. Environmental factors were compared areally and temporally. Complete sediment analyses were made.

In the four characteristic areas discerned, salinity and pH had narrow ranges, 31.9 ± 0.2% and 8.0 ± 0.2, respectively. *Outer Harbor* had the least environmental fluctuation: depth, 11 ± 3 feet; Eh, +230 ± 10 mv; sediment median diameter, 44 ± 12 microns. Nematodes (90%) and oligochaetes (9%) dominated the meiofauna in all samples. An average of 8.8 molluscan species occurred per grab. *Inner Harbor* had uniform depths of 10 ± 3 feet and sediment median diameter of 28 ± 7 microns, but Eh (often negative) showed average fluctuations from +65 to +120 mv. Nematodes, oligochaetes and harpacticoid copepods in variable small proportions comprised the meiofauna. *Vineyard Sound entrances* had uniform sands, median diameter 300 μ, depths, 1 to 10 feet, and Eh +210 to +300 mv. Amphipods composed the greater portion of a varied fauna. Channels were characterized by depths of 1–14 feet, Eh +70 to +470 mv., and sediment median diameter 38 to 552 μ. Dominant taxa (nematodes, amphipods and oligochaetes) varied widely in number geographically. Only 4.3 species of mollusks per grab occurred in deeper portions of the last three areas.

Preliminary results indicate that when environmental parameters are uniform areally, faunal abundance and gross composition vary little geographically, but individual stations show great diversity of species. When one parameter fluctuates by half an order of magnitude, faunal number and composition vary widely, but station diversity is low.

Supported by Grant GB-561 from the National Science Foundation, and Grant NR 104-747 from the Office of Naval Research.

GENERAL SCIENTIFIC MEETINGS

AUGUST 24-27, 1964

Abstracts in this section are arranged *alphabetically by authors*. Author and subject references will also be found in the regular volume index, appearing in the December issue.

PAPERS READ

Relation of hyperpolarizing response to potassium conductance in internally perfused squid axons. W. J. ADELMAN, JR. AND F. M. DYRO.

Cleaned isolated squid giant axons were internally perfused with an artificial cytoplasm solution having concentrations as follows: potassium, 93.3 mM; sodium, 20 mM; chloride, 20 mM; sulphate, 43.6 mM (isotonicity maintained with glucose). One hundred to 250 mV. hyperpolarizing responses were obtained upon passing an hyperpolarizing constant current of from 10 to 300 msec. duration from an internal axial wire to external electrodes whenever the axon was externally bathed in artificial sea water containing 100 mM potassium. Such responses usually showed very long latencies (as much as 100 msec.), and remained on as long as current was passed. Voltage clamping such axons with the holding potential set equal to the resting potential (−10 mV.) indicated that the instantaneous current response to hyperpolarizing pulses had a slope of about 12 mmho./cm.^{2} and was highly linear. This relation changed with a time constant of about 5 to 10 msec. to an N-shaped curve with a negative slope in the potential region of from −40 to −80 mV. This curve was shown to be distinct from the sodium current curve. This relation was also obtained upon clamping with the holding potential set in the −60 to −90 mV. range. In this case the steady-state current voltage relation showed a zero current crossover at a membrane potential equal to the resting potential. The instantaneous current-potential relation after a 10-msec. pulse was highly linear, and all families of these instantaneous currents for potentials more negative than the resting potential converged at the resting potential. Such evidence suggests that (1) the hyperpolarizing response is predicted from the steady-state current voltage relation; (2) the steady-state current is a potassium current; (3) the resting potential is determined by the steady-state current voltage relation.

Studies on the subcellular sites of protein synthesis in goosefish islet tissue. G. ERIC BAUER, GORDON LESTER AND ARNOLD LAZAROW.

Following the *in vitro* incubation of islet tissue with leucine-H^3 for 15 to 120 minutes, the tissue was homogenized in 0.25 M sucrose and fractionated into nuclear, secretory granule, mitochondrial, microsomal, and cell supernatant fractions. Proteins were then precipitated with TCA and extracted with acid alcohol; the specific activities (cpm/mg. protein) of these extracts (AASF) were compared.

The specific activities of AASF proteins of all cell fractions increased approximately linearly during incubation (15, 30, 60, and 120 minutes); the microsomal AASF had the highest specific activity at all time periods. The corresponding pattern of glucose-C^{14} carbon incorporation into the subcellular fractions differed from this in that there was an apparent lag of incorporation into microsome and supernatant fractions.

Electrophoresis of the microsomal AASF on acrylamide gels showed the presence of protein bands corresponding to both insulin and glucagon. The specific activities of these microsomal proteins were many times greater than those of the secretory granule AASF. This supports the view that these proteins have a microsomal site of synthesis.

The microsomes were subfractionated into four major particulate components by centrifugation on a discontinuous sucrose gradient (0.5–1.4 *M*); these subfractions differ from one another with respect to both their enzyme activities (phosphatases) and leucine-H^3 incorporation. The secretory granules were subfractionated into three components by centrifugation on a discontinuous sucrose gradient (1.4–1.9 *M*), or on a continuous sucrose gradient (1.5–2.0 *M*).

Characterization of the microsomal and secretory granule subfractions by gel electrophoresis and electron microscopic analysis is in progress.

Aided by U.S.P.H.S. Grants AM 6049 and AM 5127.

Spinal and medullary nuclei controlling electric organ in the eel, Electrophorus. M. V. L. Bennett, M. Giménez, Y. Nakajima and G. D. Pappas.

Spinal electromotoneurons and the large medullary neurons innervating them were studied by intracellular recording. In each group spontaneous organ discharge is preceded by a single spike arising abruptly from a level baseline. Thus, these nuclei relay "command signals" from an as yet unidentified higher level. Each "command" excites motoneurons supplying both main and Sachs organs, but only the latter gives a near maximal response to low frequency volleys. In the main organ p.s.p.'s show much temporal facilitation, and maximal activation occurs in response to high frequency volleys. Control over small, presumably sensory pulses and large, offensive-defensive ones is by frequency of "command" rather than by separate pathways. In both nuclei cells are electronically connected since antidromic stimulation produces graded depolarizations with the same latency as antidromic spikes. Electrotonic connections should function to increase synchronization. Electron microscopy of the spinal cord shows regions of contact between electromotoneurons and descending fibers where extracellular space is obliterated. These structures have been shown in numerous other cases to be associated with electrotonic transmission. Thus, the motoneurons are connected to each other *via* presynaptic fibers rather than directly. In normal responses the medullary relay becomes active synchronously and "commands" arrive at anterior regions of the cord earlier. Graded spinal and peripheral delays compensate for differences in conduction time and lead to synchronous organ discharge. The anatomy indicates transmission from descending fibers is electrical and therefore essentially without delay. Thus, spinal compensatory delays must be due to conduction time in terminal branches. In agreement fiber responses can be recorded at a given level of the cord with intermediate delays, and one fiber can be activated by stimulation of others at a latency consistent with propagation back from the motoneurons.

Supported by grants from NIH (B 3728, NB 02270-02 and 2B 5328 (R1)) and NSF (G-19969) to H. Grundfest.

Effects of D-malate on production and activity of L-malate dehydrogenases in developing sea urchin embryos. Reinhart B. Billiar, Joanne C. Brungard and Claude A. Villee.

Sea urchins and other echinoderms have five electrophoretically separable L-malate dehydrogenases (MDH) in adult tissues and in unfertilized eggs. The number decreases to three in preparations from whole embryos four hours after fertilization. When large and small blastomeres from these embryos are separated by centrifugation in a sucrose gradient, the small blastomeres have three and the large blastomeres two bands of MDH activity which can be separated by microelectrophresis on polyacrylamide gel and revealed by staining with nitro blue tetrazolium. It was of interest to determine whether this pattern of MDH development could be altered by growing embryos in sea water containing D- or L-malate. Preliminary experiments showed that D-malate inhibits fertilization and development at concentrations of 10^{-1} and 10^{-2} *M* but at 10^{-3} *M* fertilization and development proceed normally. In contrast, L-malate at 10^{-2} *M* permits normal fertilization and development and even at 10^{-1} *M* more than half of the eggs are fertilized and the fertilized eggs develop normally.

In a series of experiments, embryos were grown for varying lengths of time in sea water, in 10^{-2} *M* L-malate in sea water or in 10^{-3} *M* D-malate in sea water. In all of these, fertilization and development were normal. Embryos developing in D-malate for four hours had bands of L-malate dehydrogenase activity identical with the sea water controls or those grown in L-malate. However, those grown in D-malate for 6 or 12 hours had one additional band of L-malate dehydrogenase. Kinetic studies of MDH activity, measuring the reduction of DPN or its analogues in a Beckman spectrophotometer, revealed no differences in the properties of the enzyme preparations from embryos grown in D-malate, L-malate, or sea water alone. The addition of D-malate at concentrations ranging from 1.67×10^{-3} to 1.67×10^{-2} *M* to the reaction mixture in the cuvette led to an inhibition of L-malate dehydrogenase activity.

A method of rating sea urchin sperm motility. JOSEPH M. BRANHAM.

Microscopic examination is not very reliable for rating sperm motility. This method is affected by subjective error and imprecision, and it is known that motility (or respiration) is influenced by O_2 concentration, CO_2 concentration, pH, heat, light and proximity of surface. The method reported here for rating *Arbacia* sperm motility avoids some of these problems.

Living sperm sedimented faster than sperm from the same suspension killed with formaldehyde when both were centrifuged at low speeds. Perhaps sperm became oriented head downwards, in which case swimming sperm would arrive at the bottom of the tube before non-swimming sperm. Sperm from the centrifugal pellet were microscopically more active and had a higher fertilizing capacity (per sperm) than sperm remaining in the supernatant. The difference between the sedimentation rates of live and dead sperm diminished to zero as sperm aged and became microscopically immobile. Live sperm, immobilized at pH 6.5 or with high CO_2 concentration, sedimented at the same rate as formalin-killed sperm.

This difference between the sedimentation rates of live and dead sperm was used as a measure of motility. Part of a sperm suspension (about 20 million sperm/ml.) was killed with 0.02% formalin. The number of sperm remaining in the supernatant was determined after 15-ml. portions of live and killed suspensions were centrifuged horizontally at 200 *g* for 20 minutes in conical tubes. Five-ml. aliquots of supernatant were withdrawn from 20 mm. above the bottom of the tube. The optical density (420 mμ) of the original suspensions and the centrifuged aliquots were compared. Motility was expressed as the change in the optical density of live sperm minus the change in optical density of dead sperm after centrifugation. Differences of from 3 to 41 Klett units (optical density) were observed (average 13) in 54 fresh sperm samples. High values corresponded to high motility, as seen in the microscope, while low values showed low motility.

Supported by a Faculty Summer Research Award from the Lalor Foundation.

Senescence of Arbacia sperm. JOSEPH M. BRANHAM.

The exhaustion of energy reserves has long been the main explanation for sperm senescence. It was, however, early recognized (by Germmill in 1900) that the rate of motility of sperm was not clearly associated with the rate of loss of fertilizing capacity. The relationship between the loss of motility and loss of fertilizing capacity was investigated by using slow speed centrifugation for determining the motility status of a sperm suspension (see preceding abstract).

It was observed that sperm concentration decreased with time at between a quarter of a million cells and five million cells lost per ml. per hr. Since the suspensions were stirred before each test, this decrease apparently represents cytolysis. Therefore, fertilizing capacity was determined in terms of the concentration of sperm at the time of the test.

Sperm were found to lose fertilizing capacity and motility at about the same rate. Fertilizing capacity, however, approached zero more than 20 hours after apparent motility was lost (20–30 million sperm/ml.). The fertilizing capacity could be extended for more than 20 hours beyond controls, by suppressing motility at low pH (6.5–7.0) or with high CO_2 concentration. Conversely, aging was hastened by aeration or keeping the pH adjusted to that of sea water (8.0).

It is concluded that the loss of fertilizing capacity is associated with the exhaustion of energy reserves. Sperm apparently maintain some energy reserve after they become immotile and this reserve is activated under the condition of fertilization. Dilution effects or stimulation by egg substances could account for the reactivation of immotile sperm.

Supported by a Faculty Summer research award from the Lalor Foundation.

Effects of temperature on reaggregation of sponge cells (Haliclona oculata and Microciona prolifera).

VICKI BUCHSBAUM.

Pieces of *Haliclona* were placed in an aerated constant-temperature bath to determine the effect of temperature on subsequent reaggregation of the dissociated cells. Temperatures tested ranged from 19–36°. After 20 minutes, the sponges were dissociated mechanically, and the cells were left to reaggregate at 17°. Reaggregation by cells from sponges warmed to 25° or more was poor. Cells from sponges warmed to 32° failed to reaggregate. With longer exposures to the experimental temperatures, tolerance levels were lower.

Clumps of freshly collected *Microciona* were placed at 2°, 11°, 17° and 22°. Samples taken after 24 and 48 hours were mechanically dissociated, and the cells were left to reaggregate at all four temperatures for 24 hours. Reaggregation ability was measured by the aggregates per area (inversely proportional to cell activity) and the qualities of the aggregates. Reaggregation ability at 22° was independent of the temperature at which sponges had been kept. However, marked differences developed among aggregates formed at lower temperatures. At 11° cells from sponges kept at 2° reaggregated best; those from sponges kept at 22° were intermediate; and those from 11° and 17° reaggregated poorly. No reaggregation took place at 2°.

Microciona kept at 2° was sampled at intervals of several hours over a period of two days. The samples were dissociated and left to reaggregate at 20° for 24 hours. Rapid loss of reaggregation ability with time was shown by a sharp rise in numbers and decrease in size and quality of the aggregates formed. In contrast to intact sponges, dissociated cells left at 2° and similarly transferred to 20° to reaggregate lose this ability much more gradually.

Partially supported by NSF Summer Fellowship for Graduate Teaching Assistants, Stanford University, and NIH Training Grant 5T1 GM 535 04.

Distribution and behavior of some marine Turbellaria of the Woods Hole region.

LOUISE BUSH.

Samples from 34 stations have been examined, including locations at Great Harbor, Eel Pond, Nobska, Buzzards Bay, Hadley Harbor, Vineyard Sound, Barnstable Harbor and Sandwich. Eight species of Acoela, 20 species of Rhabdocoela and 25 species of Alloeocoela have been distinguished. Techniques used in sampling demonstrate the necessity of examining fresh material and of employing screens as fine as 64 μ.

The species taken to date may be separated into four distinct habitat groups: those found on algae in the intertidal and subtidal zone, those from the interstitial sand of beaches, those found on the surface of mud or sand at mean low water and those on or near the surface in the subtidal zone.

Structure and behavior associated with each of the four above habitats have been studied. The species found on algae are creeping types, though in addition some exhibit a tendency to swim upwards when disturbed. They are typically streamlined in shape and this suggests a semi-pelagic habit for at least part of their lives. Such forms maintain their position on the algae by means of a general sticky surface and their habit of creeping into protected areas and rounding up into a sticky ball. The interstitial sand species are typically much elongated and provided with special adhesive organs. Species living on the surface at all levels are delicate flattened types. The simplest and most primitive members of the group seem to be found at low tide level.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program

Preliminary studies on the deoxyribonucleic acids isolated from haploid and diploid cells of Arbacia punctulata. George A. Carden III and Herbert S. Rosenkranz.

The technique of cesium chloride density gradient centrifugation was used to study the base composition and macromolecular structure of the DNAs isolated from the sperm, eggs and somatic cells of *Arbacia punctulata.* The nucleic acids from the sperm and eggs were isolated by a detergent method. This procedure could not be used for the extraction of DNA from unfertilized eggs unless the cells were first treated with dithiodiglycol, Na$_2$EDTA and glucose. The hot phenol extraction procedure was also effective in the preparation of DNA from such cells. The DNAs from sperm, eggs and somatic cells exhibited buoyant densities of 1.700, 1.700 and 1.702 g./cm.^{3}, respectively. These densities have been reported by others to correspond to guanine-cytosine compositions of 41%, 41%, and 43%. Upon heating (100° C.) and rapid cooling, the DNAs show increases in buoyant densities of 0.015 ± 0.001 g./cm.^{3} This is taken to mean that the DNAs isolated from these cells possess double-stranded structures. The widths of the DNA bands indicate that all of the samples possess high molecular weights.

This study was aided by contract Nonr 266-89 between the Office of Naval Research, Department of the Navy and Columbia University, and by a grant from the U.S.P.H.S. (AI 05111).

Use of polished mollusk shell for testing demineralization activity of accessory boring organ of muricid boring gastropods. Melbourne Romaine Carriker and Dirk van Zandt.

Demineralization during boring of shell of prey by *Urosalpinx cinerea, Eupleura caudata* and *Thais lapillus* is effected by pressing the accessory boring organ (abo), 1.5 to 2.0 mm. in diameter, exuding a film of unidentified viscid neutral secretion, against the boring site. Little dilution by sea water occurs as the gland is closely surrounded by foot. We have developed a sensitive method for determining demineralization activity of whole excised abos. This facilitates study of the boring process and its ultrastructural effects on shell, of various reagents on the active agent, and of distribution of active agent in abo.

Large valves (CaCO$_3$ as calcite crystals in conchiolin matrix) of *Spisula solidissima* are sawed into 1.5-cm. squares with a small diamond cutting wheel held in motor-driven shaft. The nacreous surfaces of squares are ground successively on wet silicon carbide papers of grits 240, 320, 400, 600, and polished on wet silk (mesh 15,625) with alpha alumina (0.3 μ) on Buehler wheels. A scratchless, polished surface, readily etched by carbonic acid in distilled and sea water, but unaffected in the absence of atmospheric CO$_2$, results. The abo, excised from a live snail, is placed on its side on polished shell in a drop of Millipore (0.45 μ) filtered sea water, and compressed by capillarity to about half its diameter by an 18-mm. coverglass which excludes CO$_2$ and expresses secretion. Abo blood buffers carbonic acid in sea water.

Demineralization by active excised glands occurs under secretory epithelium of abo, commences at once, and continues for several hours; control tissues from other parts of the snail body do not demineralize. Abo etchings are examined by direct polarized illumination before and after shadow-casting in vacuum with chromium. Extent of demineralization is reflected in the depth of etching, and is measurable after partially dissolved loose calcite crystals are removed by collodion replication.

Supported by U.S.P.H.S. Grant DE 01870 from the National Institute of Dental Research.

A preliminary survey of the calanoid copepods of certain embayments and estuaries of Cape Cod. John C. H. Carter.

A study is being made of seasonal succession and breeding cycles of calanoid copepods in three shallow estuarine areas of Cape Cod: Town Cove, Nauset (salinity 31–32‰); Pleasant Bay (27–29.5‰); Follins Pond at the head of Bass River (19–22‰). Sampling, by means of a Clarke-Bumpus quantitative sampler with nets of No. 6 and No. 20 mesh, commenced in September, 1963, and has been continued weekly when possible. Three groups of calanoid species have been distinguished: (1) indigenous species which appear in the water column for a few weeks or months, probably entering a resting stage during the remainder of the annual cycle, *e.g.*, *Acartia tonsa*, *Eurytemora americana*, *Pseudodiaptomus coronatus*; (2) species which invade seasonally from the sea and breed successfully, *e.g.*, *Pseudocalanus minutus*, *Temora longicornis*, *Paracalanus parvus*, *Centropages hamatus*, *Tortanus discaudatus*; (3) invading species which fail to reproduce and rapidly disappear, *e.g.*, *Calanus finmarchicus*, *Metridia lucens*. Most species found were common to all three areas. Copepod production was relatively poor except during the summer outbursts of *A. tonsa*. *P. coronatus* was also an early summer dominant in Pleasant Bay although scarce in Town Cove and Follins Pond, suggesting a salinity optimum in Pleasant Bay. While poor feeding conditions probably inhibit productivity seasonally predation may also be important. In Town Cove, the hydromedusa, *Rathkea octopunctata*, bloomed in May and early June and all copepods disappeared. In Pleasant Bay and Follins Pond, *Aurelia aurita* was extremely common from March until late June and the two copepod populations then dropped to their lowest levels. Consequently no spring bloom of *Acartia clausi* occurred in any area. The ctenophore, *Mnemiopsis*, appeared in Follins Pond during September and probably contributed to the rapid decline of *A. tonsa*. During winter and spring the carniverous copepod *Tortanus discaudatus* was present in all areas, and it is possible that predation by this animal was a major factor in reducing grazing populations.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

The shark as an experimental organism. C. Lloyd Claff and Armand A. Crescenzi.

In the course of four months' study at the Lerner Marine Laboratory, Bimini, Bahamas, this year, as well as studies at Woods Hole this summer, two extremely interesting facts were discovered about the shark's heart. First, we discovered that if we carefully dissected out a shark's heart, and fixed it in 10% formalin for ten days, we could then take it out, connect it by tubing to a 30-inch head of water, and after assisting it for a few strokes by compressing the ventricle of the heart by hand, the heart would fill and empty by itself. We have assumed that this phenomenon is the result of mechanical differential hydrodynamic pressures, due to the shape, structure, and valving of the two-chambered shark heart—the heavy-walled ventricle and the thin, paper-like atrium.

The second fact is that under the conditions of our experiments, *i.e.*, with a shark out of water on an operating table, there was a distinct negative phase of from three to five mm. of mercury in each complete cycle of the heart-beat, measured by a mercury manometer.

When we reported this to Dr. Robert Mathewson, Director of the Lerner Laboratory, he produced an abstract, written a month earlier by Dr. Fred Sudak, working at the same Laboratory, who, with the use of pressure transducers had discovered the same phenomenon.

These two observations: the pumping of the isolated shark heart, and the negative phase in the heart-beat, suggest to us that others may find the shark an interesting and profitable source of study. We think we have finally found a useful role in research for the much maligned shark.

Supported by the Single Cell Research Foundation, Inc., Randolph, Mass.

Evidence for the presence of "renin" in kidneys of marine fish and amphibia. Gerald M. [illegible] and Gabor Kaley.

The polypeptide hormone, angiotensin, apparently has two important physiological roles in mammals: (1) elevation of blood pressure, and (2) stimulation of aldosterone secretion. Angiotensin is produced by the action of the renal enzyme, renin, on its plasma substrate, angiotensinogen, which is a component of the alpha-2-globulin fraction of plasma. Although the renin-angiotensin system has been studied extensively in mammals, little work has been completed in lower vertebrates. A recent report suggested that renin is present in at least one species of marine teleost, *Paralichthys* (flounder) and one species of fresh water reptile, *Chrysemys* (turtle). In an effort to extend these observations, kidneys of several other types of marine fishes and one species of amphibia were examined for the presence of renin. Renin was assayed by the injection of partially purified kidney extracts into rats whose blood pressure was recorded. Extracts of kidneys also were incubated with homologous, as well as heterologous, plasmas and the resultant pressor substances were similarly assayed and were compared to synthetic angiotensin standards. Our results indicate that kidneys of *Centropristes* (sea bass), *Anguilla* (eel), and *Paralichthys* (flounder) contain amounts of renin comparable to that of control rat kidneys. In addition, kidneys of *Rana catesbeiana* (bullfrog) possessed renin activity which approximated that found in mammals. Although the renin-renin substrate system was found to be class-specific, no species specificity was observed among the three marine teleosts studied. Criteria for angiotensin production in all instances included: (1) inability to produce pressor substances with boiled kidney extracts, (2) inhibition of pressor activity by incubation with trypsin, (3) persistence of pressor activity in dibenzyline-pretreated rats, and (4) measurement of plasma angiotensinase activity. A large number of abundantly granulated juxtaglomerular cells, the site of renin synthesis and storage in mammalian kidneys, were noted upon histological examination in the sea bass kidney.

This work was completed in the Comparative Physiology Training Program under the support of NIH GM 1030.

Gas secretion in teleost swimbladder, Fundulus and Opsanus. EUGENE COPELAND.

The secretory epithelia of swimbladders from *Fundulus heteroclitus* and *Opsanus tau* were fixed in buffered glutaraldehyde and osmic acid. The fine structure of normal bladders and of bladders induced to activity was studied. Increase in secretory activity is characterized by loss of glycogen particles and an increase in villi at the gas surface of the cells. Complex bodies having granular and fibrillar configurations of varying density are seen. In *Opsanus* these are frequently seen as ovoid bodies with a fibrillar spindle in the center surrounded by a granular or crystalloid externum. Endoplasmic or cisternal elements are found throughout the cell, but their arrangement near the surface (*Fundulus*) strongly suggests a collecting and transport mechanism terminating at the lumenal surface of the epithelium. The bases of the cells are thrown into complex interdigitating folds. The folds appear to have rows of vesicles associated with them (osmic fixation). However, more recent study (glutaraldehyde fixation) reveals that the vesicles have formed from interdigitating cell membranes that were not preserved by osmic acid. Though not conclusively demonstrated, it is quite possible that the secreted gas is released in the form of minute oxygen bubbles.

Supported by grants from the National Science Foundation (GB-676) and the NIH (GM 06836).

Salt-absorbing cells in gills of crabs, Callinectes and Carcinus. EUGENE COPELAND.

The gills of *Callinectes* and *Carcinus* were perfused with buffered osmic acid and buffered glutaraldehyde. The fine structure of the salt-absorbing cells (identified by affinity for silver) in the respiratory leaflets of fresh-water-adapted animals was studied. The patch of salt-absorbing cells hypertrophied markedly (*Callinectes*) on adaptation to fresh water. A network of tubules associated with the patch also hypertrophied. The cells of *Carcinus* are located peripherally on the leaflets and those of *Callinectes* more closely about the afferent vessel. Otherwise, both are similar. The salt-absorbing epithelium is one cell layer in thickness and the cuticular border is thrown into evenly spaced folds that lay in random whorls. Pynocytosis apparently occurs at the bases of the folds. The major share of the cell is occupied by mitochondria closely associated with double membranes of interdigitating folds of the cells. Considerable amounts of rough endoplasmic reticulum and Golgi groups are seen from the level of the basal folds (cuticular) up to the level of the nuclei. What appears to be a network of smooth endoplasmic reticulum (osmic fixation) leading to the plasma surface of the cell proves to be instead interdigitating folds (glutaraldehyde fixation) which break down with osmic fixation. There are also numerous microtubules that run from near the cuticular surface to near the plasma surface. It is not clear what their function may be. The mitochondrial association with the numerous double membranes of the interdigitating folds is considered significant.

Supported by grants from the NIH (GM 06836) and the National Science Foundation (GB-676).

Relationship of mesh opening to faunal counts in a quantitative benthic study of Hadley Harbor. ANDREW L. DRISCOLL.

Study of benthic faunal populations of Hadley Harbor, Woods Hole, required determination of optimum sieve mesh opening for obtaining maximum numbers of organisms most efficiently. Sixteen $1/25\text{-m.}^2$ Van Veen grab bottom samples were washed through a series of sieves with mesh openings of 4, 2, 1, 0.5 and 0.25 mm., then sorted into major taxa and the fauna counted. Several habitats and sediment sizes ranging from well-sorted sand to silt were included in the sampling.

Of the total of 27,492 animals counted in the 16 samples, 60% were retained by the 0.25-mm. sieve, 32% by the 0.5-mm. sieve, 5% by the 1-mm. sieve and 2% by both the 2- and 4-mm. sieves. Thus, the 0.25-mm. sieve retained the maximum proportion of animals. Seven taxa made up 98% of the count (11,034 animals), which included the 4-, 2-, 1- and 0.5-mm. sieve fractions: Pelecypoda, 3%, were retained primarily on the 4-mm. sieve; Amphipoda, 17%, Ostracoda, 20%, Polychaeta, 19%, and Oligochaeta, 12%, were retained on the 0.5-mm. sieve, and Nematoda, 25%, and Copepoda, 0.2%, on the 0.25-mm. sieve. The same taxa taken on all five sieves made up 97% of the total count (27,492): Pelecypoda, 1%, Amphipoda, 9%, Ostracoda, 9%, Polychaeta, 9%, Oligochaeta, 8%, Nematoda, 57% and Copepoda, 4%. Thus, as smaller mesh opening fractions are added the faunal composition of the samples varies considerably. Nematodes make up 96% of the animals retained on the 0.25-mm. sieve and were the only animals consistently found at all stations, regardless of sediment size and other physical factors.

These results indicate clearly the value of utilizing a minimum mesh opening of at least 0.25 mm. in sieving, and disclose the wide distribution and abundance in this area of meiofauna which would have been largely overlooked by use of coarser sieves.

Supported by Grant GB-561 from the National Science Foundation.

Chloride fluxes in crayfish muscle fibers after vesiculation of the transverse tubular system and after treatment with procaine. PHILIP B. DUNHAM, JOHN P. REUBEN AND PHILIP W. BRANDT.

Total chloride determined on groups of 2 to 12 muscle fibers of crayfish walking leg is about 65 meq/kg., after correction for inulin space. About 40 meq/kg. of apparently immobile Cl remained after the preparations were soaked in Cl-free saline for up to 8 hours. Tracer analyses during washout after loading the fibers with Cl^{36} indicate that the exchangeable Cl leaves the cells with three different time constants. During the initial washing (10 to 20 seconds), which is needed to remove the Cl^{36} from the medium before counting is begun, up to 12 meq/kg. is removed. Of the remainder, 5 meq/kg. leaves with a half-time of less than one minute. The final 8 meq/kg. is removed with a half-time of 12.5 minutes.

About 13 meq/kg. of Cl enter the fibers with a half-time of 5 minutes. Procaine (0.1%), which induces spike electrogenesis while blocking contraction of the fibers, reduces the influx markedly, to about 6 meq/kg. after one hour. The influx is accelerated at least 30-fold when the transverse tubular system (TTS) is swollen, but the amount of exchangeable Cl is essentially unchanged. When the TTS is swollen, addition of procaine also reduces the rate of influx to about one third, but the influx is still about 10 times faster than in control preparations.

Contractile activity may be produced or enhanced when Cl influx is accelerated and may be blocked when influx is diminished. These correlations appear to be independent of changes in membrane potential. They suggest that movement of Cl may play a role in contraction coupling.

Supported by grants from NIH (B 3728, NB 02370-02, and 2B 5328 (R1)) and NSF (G-19969) to H. Grundfest.

A fine-structure study of the activation reactions of Nereis limbata gametes. JOHN F. FALLON AND C. R. AUSTIN.

The egg of *Nereis limbata* is shed without a jelly coat. The vitelline membrane of the egg is composed of a granular material having numerous canals extending from its inner border to, but not through, the outside surface. The plasma membrane lies directly beneath the vitelline membrane in the unfertilized egg. Irregularly spaced microvilli protrude through the vitelline membrane. The jelly precursor material, which appears as a fine thread-like substance arranged in tightly packed whorls, lies in cortical alveoli below the plasma membrane. The alveolar walls appear to be in the process of fusion and breakdown in the mature egg.

In the reacted spermatozoan, the outer acrosomal membrane and plasma membrane are fused, the acrosome is broken down, and the acrosomal rod (Fallon and Austin, 1963) is extruded from the nuclear invagination. The rod undergoes progressive fibrification and expansion in a proximo-distal direction. The fibrous acrosomal rod is covered with the inner acrosomal membrane; the latter makes contact and fuses with the egg plasma membrane. After sperm-egg membrane fusion, and for about 15 minutes thereafter, the membranes of the cortical alveoli fuse with the plasma membrane. Numerous small membrane vesicles can be seen as a result of this coalescence. The jelly precursor is thus freed beneath the vitelline membrane. The jelly reaches the outside of the egg through the pores in the vitelline membrane which contain the microvilli, creating a fountain-like effect at the surface of the egg. The blind canals of the vitellus have no function in jelly extrusion. The space formerly occupied by the jelly precursor becomes the perivitelline space in the fertilized egg. The plasma membrane of the fertilized egg is formed partly from the membrane of the cortical alveoli and partly from the plasma membrane of the unfertilized egg.

Aided by Training Grant 9 T1 HD 26-03 from the NIH.

Specific puff induction by tryptophan in Drosophilia salivary chromosomes. NINA FEDOROFF AND ROGER MILKMAN.

Analysis of induced enzyme biosynthesis has demonstrated the involvement of small molecules in the control of genetic activity. To learn more about the role of naturally occurring small molecules in gene activation, we resolved to study their effects on giant dipteran salivary chromosomes, whose puffs have been shown to be sites of intensive synthesis of RNA, the primary gene product.

One of each pair of salivary glands excised from third instar *Drosophila melanogaster* larvae was incubated in insect Ringer's solution (control), while the other was placed in Ringer's containing the test substance. Gland pairs were fixed simultaneously, lightly stained, squashed, and examined under phase. Five nuclei in each of 5–20 glands were examined. Although exposure to Ringer's itself changes the puffing pattern of the chromosomes, a clear-cut differential effect was observed after 60 minutes in Ringer's containing 0.03 *M* 1-tryptophan. A puff never observed in normal development appeared at site 68D on the left arm of the third chromosome. Methionine and tyrosine had no visible effects in similar experiments. From these results and direct pH measurements, it is concluded that the induction of the 68D puff is not dependent on pH, ionic strength, or amino acids in general.

The diversity of treatments (ecdysone, $ZnCl_2$; oxygen, mechanical injury) used by others to induce arrays of puffs, and the similarity of such arrays to those found during normal development, suggest that these treatments act through common intermediates. It is thought that the tryptophan-induced puff may be similar to the unit response produced by such an intermediate.

A comparison of soluble dogfish skin collagens. LOUIS FISHMAN AND MILTON LEVY.

The soluble collagen of the skin of the smooth dogfish *Mustelus canis* was extracted in 0.1 *M* pH 4.3 citrate after suitable washing. The supernatant after centrifuging at 12,500 × *g* was dialyzed against 0.02 *M* Na_2HPO_4. All work was done below 5° C.

Instead of the usual fibers the isolated collagen was a milk-white, rigid gel which conformed to the shape of the dialysis bag. No such isolated collagen had been encountered in our experience and further study of the physical and chemical properties was undertaken. The *M. canis* collagen was compared to other soluble fish collagens, particularly that of *Squalus acanthias.*

The phase transition temperature, which is the temperature at which the triple-stranded helical portions of the collagen molecule unfold, was established by measuring the change in relative viscosity as a function of temperature. The transition temperatures were *S. acanthias* 16° C., *M. canis* 24° C., and carp swim bladder ichthyocol 30° C.

The magnitude of the fall in viscosity at the phase transition temperature for these collagens at the same concentration was equal, indicating that the rigid gel of *M. canis* was not denatured.

The shrink temperatures of the fresh intact skins were as follows: *S. acanthias* 41–43° C., *M. canis* 49–51° C., and carp swim bladder 54° C. It was possible to measure the shrink temperature of the *M. canis* gel which was 33–35° C.

The total imino acid (proline + hydroxyproline) content of these collagens was in a linear relationship to the phase transition or shrink temperatures, as was the reducing hexose as measured by the anthrone method.

It is possible that the rigid gel state of *M. canis* skin collagen is due to greater cross-linking and warrants further study.

The cleavage schedule and the development of Arbacia eggs as separately influenced by heat shocks. W. L. M. GEILENKIRCHEN.

Between fertilization and second cleavage, *Arbacia* eggs differing in age by periods of four or five minutes were heat-pulsed for five minutes at 34.5° C. After treatment the time for the next two cleavages to occur was determined in each sample and the cleavage delay was calculated. Secondly, after about 6 hours, samples of 30 to 40 free-swimming blastulae of each treatment group were isolated and reared for three days. When the treatment occurs between fertilization and prophase of first division, it causes a delay of first cleavage in excess of the duration of the treatment itself. Maximal delay is found for treatment shortly before prophase. As the time of treatment passes this maximum, the delay decreases rapidly and becomes zero when the treatment is given shortly before cleavage. Treatment at stages between prophase of first division and prophase of second division causes a delay of second cleavage. Shocks given after the prophase, preceding second division, no longer delay second cleavage but delay third cleavage. It is clear that the system responding to shock goes through a cycle from prophase to prophase.

Heat shocks applied between 60 and 30 minutes before fertilization have no retarding effect on first cleavage. However, pulses given between 30 and 5 minutes before fertilization have an increasing delay effect on first cleavage. This last pulse causes a delay which reaches the same level as pulses after fertilization. From these data it is seen that the unfertilized egg needs 30 minutes to recover from a shock. Fertilization interferes with this repair process in the unfertilized egg and apparently causes a lengthening of the repair period.

Heat shock applied before fertilization had no effect on development. Treatment during mitosis of first and second cleavage produced disturbances of development, with the maximum effect at metaphase. Eggs heat shocked before fertilization, between fertilization and prophase of first cleavage, or between first cleavage telophase and the beginning of second cleavage prophase, developed into normal plutei.

It is apparent, that in the course of one division cycle the egg discriminates between processes for the preparation of the next division and processes involved in morphogenesis and differentiation later on. Such a discrimination I have observed also in the mosaic eggs of *Limnaea stagnalis.*

This work has been supported by a fellowship of The Netherlands Organization for the Advancement of Pure Research.

The chromosomal complement of blastomeres in Arbacia punctulata. JAMES GERMAN.

Blastulae of *Arbacia punctulata* were treated 10 minutes with Colcemide and then trypsinized in calcium-magnesium-deficient sea water. The dissociated blastomeres received hypo-osmotic treatment by addition of distilled water to the medium, following which the cells were fixed in acetic methanol and stained with orcein.

One modal number of chromosomes, 44, was found and thus represents the diploid somatic number of both sexes. Delicacy of cell membranes resulted in occasional ($<7\%$) apparently intact cells with 43–38 units. No cell with 22 or more than 44 chromosomes was found, except for very infrequent possibly tetraploid groupings.

There are two pairs of chromosomes with distinguishing structural features; the remaining members of the complement can only be grouped with some accuracy. The acrocentric Nos. 1 (arm ratio 5.1) are about 4 μ in length, the sub-mediocentric Nos. 2 (arm ratio 2.6) about 3 μ. In an occasional cell only one No. 2 was recognized. In some cells the sub-mediocentric Nos. 3, which are somewhat shorter than the Nos. 2, can be distinguished, but in most cells they can only be grouped with the other sub-mediocentric members of Group 3–7 whose length is near 2 μ. Group 8–12 consists of 10 short acrocentrics, Group 13–14 of four short sub-acrocentrics, and Group 15–22 of 16 short mediocentrics or sub-mediocentrics. Sex elements, if present, were not identified, although a difference in any pair other than Nos. 1 of 2 would be recognized with difficulty.

Forty of the chromosomes unfortunately are small, but because of the two large readily identifiable pairs, the complement can be used for certain studies of chromosomal replication or the effects of breaking agents. The ease of handling of these easily available, rapidly dividing embryos, as well as the extensive information now available about mitosis, metabolism, and biochemistry during development in this particular species, stimulate further exploration of chromosomal structure and mechanisms.

Supported by NSF grant GB-1868.

Heat denaturation studies on Arbacia punctulata nucleoproteins. MARTIN A. GOROVSKY.

Bonner and Huang (1963) have suggested that a fraction of DNA not complexed with histones in pea embryo chromatin is responsible for DNA-dependent RNA synthesis *in vivo*. The sperm (a relatively inactive cell in terms of RNA synthesis) and the gastrula (consisting of highly active cells) of *Arbacia punctulata* have been examined to determine whether a similar fraction exists in animal cells. The hyperchromicity accompanying heat denaturation of "chromatin" was analyzed.

Whole washed sperm were extracted with cold dilute saline citrate (0.015 *M* NaCl, 0.0015 *M* Na_3 citrate). A crude preparation of gastrula nuclei was similarly extracted and filtered through Sephadex G-200 to remove the echinochrome. Heat denaturation (melting) of formaldehyde-treated extracts was carried out in the electrically-heated cuvette chamber of a Beckman DU spectrophotometer. Reactions run without formaldehyde showed qualitatively similar melting profiles, but actual temperatures were 30° higher.

Isolated sperm DNA (prepared according to Marmur, 1961) gave a sharp melting profile with a half melting point (Tm) of 49–51° C. and a hyperchromicity of 45–50%. Sperm "chromatin" showed a single step profile with a Tm of 75–80° C. and a hyperchromicity of 30–35%. Gastrula "chromatin" gave erratic patterns but always melted with Tms lower than that of sperm (50–72° C.) and hyperchromicity of 9–17%.

The irregular results with the gastrula make it difficult to decide whether the nuclei of these active cells contain DNA not complexed with protein. The single step melting profile of the sperm extracts is consistent with the observations of Huang, Bonner and Murray (1964) on calf thymus nucleohistones, inasmuch as the inactive cell shows a greater degree of stabilization of the DNA helix to heat denaturation. However, the histone of the sperm is arginine-rich (Hamer, 1955) while preliminary cytochemical observations indicate the presence of predominantly lysine-rich histone in gastrula nuclei. Thus, the histone type (arginine-rich as opposed to lysine-rich), which appears to maximally stabilize the DNA in *Arbacia,* is quite different from that which provides maximum stability in the calf thymus material. This may indicate that the different stabilizing abilities of different histones are not dependent on the nature of their basic amino acid content, but on some other parameter, perhaps secondary structure (Huang *et al.*, 1964).

Aided by Training Grant 9T1 HD 26-03 from the National Institutes of Health.

Temperature characteristics of excitation in squid axon. RITA GUTTMAN AND ROBERT BARNHILL.

Temperature characteristics of excitability in the squid giant axon were investigated, utilizing the double sucrose gap technique. Strength-duration curves were established for square wave current pulses of durations ranging from 10 μsec. to 10 msec. at temperatures ranging from 5° C. to 35° C. Threshold membrane current and voltage were studied, and resting potentials were monitored throughout.

The Q_{10} obtained for rheobasic current was about 1.9 as compared to the value of 2.3 obtained with cruder methods in 1961. There is a relatively slight effect of temperature at short current pulses. Threshold membrane voltage was studied by superimposing the largest local response trace upon the action potential at threshold and measuring the voltage at the point of deviation, but these records have not as yet been analyzed. The time constant of the excitation process was decreased by increasing temperature and gave a Q_{10} of about 1.8.

FitzHugh is in the process of investigating the theory with regard to the effect of temperature upon excitation in terms of the Hodgkin-Huxley equations with computers. The interpretation of our results will be delayed until his detailed investigation is completed. However, it is indicated that our results are probably in accord with the Hodgkin-Huxley formulations, although there seem to be some minor differences not as yet explained.

Aided by National Science Foundation Grant GB-1463 and Laboratory of Biophysics, NINDB, NIH.

A qualitative study of the hatching enzyme in the sea urchin, Arbacia punctulata. ROBERT L. HALLBERG.

In *A. punctulata* the ciliated blastula breaks out of the fertilization membrane by digestive action of a "hatching" enzyme (Kopac, 1941; Yasumasu, 1960). Current studies on sea urchins (Gross and Cousineau, 1964) suggest that protein synthesis is independent of immediate genomic control prior to gastrulation. Therefore, two possible explanations for the appearance of hatching enzyme activity are: (1) *de novo* synthesis on preformed messenger RNA; or (2) activation of an inactive protein already present. This study sought to determine which alternative might obtain.

A crude solution of the hatching enzyme was prepared by culturing dilute embryo suspensions until the first signs of hatching appeared. The embryos were then resuspended in veronal-HCl-buffered artificial (MBL formula) sea water at pH 8.2 as dense suspensions and allowed to hatch. After 95% had hatched, the embryos were spun down at 12,000 *g* for 10 minutes. The supernatant was then concentrated by dialysis against veronal-HCl-buffered PVP at pH 8.2. The resulting solution digested fertilization membranes of 4- and 8-cell embryos. This crude preparation after fractionation by Sephadex G-100 filtration showed membrane-digesting activity localized in the single, unretarded 280 mμ absorbing peak. In agar gel diffusion tests with an anti-whole unfertilized egg rabbit serum, crude and gel filtered preparations gave a major and two minor precipitin bands. The major band did not appear to join any band produced by homogenates of hatched embryos.

The 12,000 *g* supernatant of homogenates of unfertilized eggs showed no membrane digesting action. However, the same supernatant filtered through G-100 Sephadex showed membrane-digesting activity in the unretarded, 280 mμ absorbing fraction (three independent experiments). Treatment of early embryos with the final fraction delayed hatching and completely arrested development at early gastrula.

These initial findings suggest that the hatching enzyme is already present in the unfertilized egg, inhibited in some manner, requiring a change in internal environment to become activated.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

RNA metabolism during maturation and early development in Asterias forbesi eggs. GEORGE HAND, JR. AND RACHELE MAGGIO.

A 30-minute pulse of 0.4 μC/ml of tritiated uridine (7.69 mC/μM) was given to unfertilized and fertilized eggs during maturation and early development. RNA was extracted and purified by the phenol procedure, and the distribution of radioactivity into RNA fractions was investigated after separation by centrifugation on a sucrose gradient. Both in unfertilized and fertilized eggs, a slight incorporation is observed all throughout the gradient with small but definite peaks between 26 and 18S; most of the radioactivity appeared from the 4S region to the top of the gradient. In later stages of development (blastula), incorporation is substantially increased also in the heavy regions of the gradient, but non-coincident with ribosomal peaks.

Eggs were pulse-labelled for 10 minutes with 2.5 μC./ml. of tritiated uridine (4 mC./ μM). Rate of total uptake by eggs and of incorporation into RNA was estimated, using the filter paper disk method of Mans and Novelli (1960). A small incorporation in DNA was detected only in later stages of development.

The following differences have been found between eggs undergoing maturation while unfertilized and those in the fertilized state. When fertilized within a few minutes after breakdown of germinal vesicle, eggs exhibit smaller incorporation of uridine into RNA than their unfertilized counterparts. Indeed, at this stage, specific activity is higher in total RNA extracted from unfertilized than from fertilized eggs. A slight but significant increase of incorporation into RNA takes place in fertilized eggs in the course of maturation; on the other hand, considerable scattering of the experimental points is observed in unfertilized eggs. In the latter, rate of incorporation is dependent on changes in total uptake. Such dependence is not observed in fertilized eggs. It is suggested that regulation of RNA metabolism is established shortly after fertilization.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health. R. Maggio, Lalor Foundation Fellow.

Hyaline membrane lysis in Mytilus edulis eggs. Stephen D. Hauschka.

Digestion of the hyaline layer surrounding *Mytilus* eggs (Dan, 1962) has been studied biochemically and on a fine structure level in an attempt to characterize the composition of the layer. Eggs collected from minced ovaries were lysed by two washes in distilled water. Membranes were then suspended in 0.5 M NaCl, 0.0025 M $MgCl_2$ buffered at pH 8.2 with Tris and separated from most cytoplasmic debris by ten gentle washes, each followed by one minute centrifugation at 200 g. Electron microscopy of glutaraldehyde-fixed membranes revealed an exterior jelly layer, hyaline material surrounding the microvilli, and cortical granules.

The hyaline layer was rapidly digested by purified lysin from sperm (Hauschka, 1963) as well as by pronase and crystalline trypsin, but not by lysozyme. Membranes were stable at acid pH, but were destroyed instantaneously by pH above 10.5. They were also destroyed by 8 washes in distilled water or by prolonged treatment with 1 M NaCl. Cysteine (0.2 M, 0.5 M NaCl, pH 8.2) caused rapid dissolution of the hyaline material, as did formamide (25%) and 5 M urea. The hyaline layer was not solubilized by a number of common organic solvents. Pre-incubation of the membranes in rabbit antisera prepared against whole eggs protected the hyaline material from digestion by the lysin from sperm, enzymes, and formamide. Protection against formamide indicates the antibodies may be binding the hyaline material together, rather than acting as non-specific blocking agents as had been previously assumed. Antisera prepared against sperm had no protective effect.

Electron microscope observations of the digestion process confirmed Dan's findings, indicating that only the hyaline layer was removed by the lysin from sperm. Its disappearance appeared to occur in patches rather than uniformly from the hyaline surface. Preliminary observation of the action of formamide indicated that it removed the exterior jelly layer prior to causing a change in the hyaline material.

Aided by Training Grant 9 T1 HD 26-03 from the NIH.

Mechanical properties of the starfish öocyte during maturation divisions. Yukio Hiramoto.

Some mechanical properties of the öocyte of the starfish, *Asterias forbesi,* were determined during progress of the maturation divisions. The relative stiffness of the öocyte was expressed by the distance between two plates parallel to each other when the öcyte was compressed between them with a definite force. The stiffness decreases after the disappearance of the germinal vesicle, increases before the onset of the formation of the first polar body, and decreases during and after the polar body formation. The stiffness increases again before the formation of the second polar body and reaches a maximum about the onset of the second polar body formation, followed by a decrease during and after the formation. These stiffness changes during the maturation divisions are similar to those of the sea urchin egg during mitotic division (Hiramoto, 1963). The stiffness of the öocyte increases by the application of heavy water, probably owing to gelation of the protoplasm. A transient increase in the stiffness was observed at the time of the formation of abortive polar bodies in D_2O-sea water. It is suggested that the surface forces of the öocyte change rhythmically during the meiotic cycle and that the polar body is extruded as a result of tensional differences that develop in cortical cytoplasm and/or surface membranes of the polar body, the main cell body and the intervening furrow region.

Work carried out during tenure of a Lalor Foundation Fellowship.

Adenylate kinase and ATPase activities in Spisula and Asterias eggs. Barbara A. Horwitz and Leonard Nelson.

The effects of fertilization on enzymes concerned with ATP hydrolysis and synthesis were investigated. Eggs were disrupted by treatment with an ultrasonic disintegrator (8 to 15 seconds) and the resulting homogenates were incubated at 26° C. for all assays.

Maximum ATPase activity, measured in Tris buffer (pH 7.45) containing 10^{-3} M $MgCl_2$, was found to be relatively low (0.25 μm P_1/mg. protein/hr.) in unfertilized eggs of *Asterias forbesi,* compared to the values obtained for unfertilized ova of *Spisula solidissima,* the latter being approximately 9 times greater. Fertilization of *Asterias* eggs is followed by a 150% increase in activity; however, the enzyme activity of fertilized *Spisula* eggs is not significantly different from that of the unfertilized eggs.

The pattern of adenylate kinase activity after incubation of the homogenates in Tris buffer (pH 8.5) with 10^{-3} M $CaCl_2$ was similar to that observed in the ATPase assays. The activity of adenylate kinase is 1.5 times greater in unfertilized egg homogenates of *Spisula* than in those of *Asterias.* After fertilization, however, the activity of the *Asterias* egg homogenates increases to a value which is 3.5 times that of the unfertilized eggs, while no significant difference was observed between fertilized and unfertilized *Spisula* eggs.

The fact that the two enzymes behave in a parallel fashion after fertilization in the particular organism is not surprising since both enzymes are concerned with a compound which is involved not only in the energy metabolism of the cells, but also in nucleic acid and protein synthesis. The increased activities seen after fertilization of *Asterias* eggs suggest that the low activities observed in the unfertilized ova may be insufficient for the metabolic demands immediately after fertilization, while the higher levels exhibited in *Spisula* eggs may be adequate. This interpretation is supported by the observation that the amino acid uptake and incorporation into proteins increases immediately after fertilization in *Spisula* ova, while a lag period is present in the eggs of *Asterias,* no change being seen until after formation of the first polar body (Tolis and Monroy, 1963). A complete understanding of these results, however, must await analysis of the adenine nucleotide pool.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

Rates of oxygen consumption of Arbacia, Asterias, and Spisula eggs. Barbara A. Horwitz and Leonard Nelson.

The rates of oxygen consumption of unfertilized and fertilized eggs of *Arbacia punctulata, Asterias forbesi* and *Spisula solidissima* were determined with a rotating platinum oxygen-electrode in a partially closed reaction cell. All measurements were made in filtered sea water at 15° C. Unfertilized eggs of *Asterias* have the highest rate of oxygen uptake (20×10^{-7} μm, O_2/min./egg) while *Spisula* ova have the lowest (1.0×10^{-7} μm O_2/min./egg), those of *Arbacia* being intermediate in value (7.0×10^{-7} μm O_2/min./egg). Fertilization of *Arbacia* and *Asterias* eggs, which was carried out in the reaction vessel of the polarograph, is accompanied by an immediate but transient increase in the rate of oxygen uptake. This increase, which lasts approximately 1½ minutes, is greater in the eggs of *Arbacia* than in those of *Asterias*. The oxygen consumption of fertilized *Asterias* eggs returns to a rate comparable to that of the unfertilized eggs within three minutes after fertilization; the rate of oxygen uptake of *Arbacia* eggs also falls, but to a level which is still significantly higher than that of the unfertilized eggs. Similer observations have been reported for other sea urchin eggs (Ohnishi and Sugiyama, 1963).

The transient increase in the rate of respiration seen upon fertilization, which cannot be explained by the presence of activated sperm nor as an artifact resulting from acid production after fertilization (which occurs after the increase in oxygen consumption), is suggested to be associated with the lifting of the fertilization membrane. Successful fertilization of *Spisula* eggs could only be accomplished in egg suspensions too dilute to provide accurate data by this technique; thus, the rate of oxygen uptake immediately after fertilization could not be measured. However, measurements obtained 10 minutes after insemination were not significantly different from those of the unfertilized eggs.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

Preliminary determinations of the DNA base composition by specific densities in the haploid and diploid generations of a red alga, Griffithsia globulifera. STEPHEN H. HOWELL AND MAIMON NASATIR.

Haploid and diploid *G. globulifera* plants collected at Woods Hole were sexed and prepared as acetone powders. DNA was extracted by the hot phenol method modified from Scherrer and Darnell (1962). The extracted DNA in 0.02 *M* phosphate 0.001 *M* citrate buffer with 2% Dupanol was precipitated with cold ethanol. The DNA was washed thoroughly with cold ethanol, dissolved in 0.015 *M* NaCl, 0.001 *M* citrate solution, and centrifuged at 32,000 *g* at 2° C. The supernatant fraction was precipitated with ethanol.

A characteristic nucleic acid absorption spectrum was given by this extract. The orcinol reaction indicated that *ca.* 70% of the UV absorbing material was RNA, and the diphenylamine reaction indicated that 10% of the UV absorbing material was DNA. It was not possible to account for 20% of the UV absorption.

These extracts from the male and female haploid and the diploid plants were dissolved in an optically pure solution of CsCl with a density of 1.70 g./cm.^{3} and centrifuged in the Beckman Model E ultracentrifuge at 44,770 rpm at 20° C. for *ca.* 24 hours. The UV absorbing bands were photographed, and the banding patterns were measured with a Joyce-Loebl Mark III B double beam microdensitometer. From the position of the *G. globulifera* DNA, relative to the reference *Micrococcus* DNA, the specific density of the *G. globulifera* DNA was calculated.

The *G. globulifera* DNA bands from the ultracentrifuge were weak and heterodispersed; however, the banding pattern indicated the specific densities of the DNA as follows: female plant, 1.7152 g./cm.^{3}; male plant, 1.7155 g./cm.^{3}; diploid plant, 1.7203 g./cm.^{3}. These specific densities, which represent approximately 56% GC, if the DNA is double-stranded, suggest that there is conformity in the gross base composition of DNA in the plants of the haploid and diploid generations of *Griffithsia globulifera.*

We gratefully acknowledge the generous help of Dr. Herbert S. Rosenkranz. This work was supported in part by a grant from the National Science Foundation (NSF-GB36).

The effect of inhibition of energy metabolism and protein synthesis on sponge cell aggregation. TOM HUMPHREYS AND MARGARET UEHARA.

Beginning with Galtsoff in 1925, various workers have studied the effects of inhibitors on cell aggregation. However, only in the aggregation of sponge cells chemically dissociated in calcium- and magnesium-free sea water has the system been characterized sufficiently that the defect(s) preventing normal aggregation are known. In this system the defect has been shown to be the absence of a single factor which is apparently an intercellular material that binds the cells together. This was demonstrated by showing that sponge cells (*Microciona prolifera* and *Haliclona occulata*) inhibited from aggregation by low temperature adhere normally when a species-specific factor, which was washed from the cells during dissociation, was replaced. Use of other inhibitors in this system, therefore, could be very informative.

Sponge cells at 22° C., whose energy metabolism was inhibited with 2 m*M* 2,4-dinitrophenol, behaved much as cells inhibited with low temperature. Again the factor was sufficient to overcome the inhibition.

In puromycin, a specific inhibitor of m-RNA directed protein synthesis, chemically dissociated sponge cells aggregated normally for 6 hours at concentrations of 50 to 200 μgm. per ml., even though incorporation of radioactive C^{14}-leucine was immediately reduced to 10% of controls. After 6 hours at 50 μgm. or more the aggregates began to degenerate and would partially (*M. prolifera*) or completely (*H. occulata*) disintegrate by 24 hours. The degeneration of aggregates after 6 hours could not be reversed by the factor. Removal of the puromycin before four hours would prevent the onset of degeneration, but removal after 6 hours could not reverse it. (Inhibition by low temperature or dinitrophenol was reversible for many hours.) The failure of aggregation after 6 hours in puromycin is the result of a generalized lethality. These results show that although some energy-requiring metabolic event is necessary for the production of the missing factor, it is not m-RNA directed protein synthesis.

Identification of lysosomes in the brains of lower vertebrates. AARON JANOFF AND C. ROBERT JONES.

Lysosomes have been demonstrated in neurons of brain and spinal cord in higher forms. However, nerve tissues of lower forms have not been extensively studied with respect to these subcellular particles. The results presented below provide biochemical and histochemical evidence for the presence of lysosomes in neurons of three cold-blooded vertebrates.

Sucrose homogenates of the brains of *Paralichthys* sp., *Centropristes striatus,* and *Rana pipiens* were separated into sub-fractions by differential centrifugation. The following fractions were obtained and assayed for lysosomal enzyme activity: whole homogenate; nuclear fraction (400 *g* × five minutes to 800 *g* × 10 minutes); granule fraction (800 *g* × 10 minutes to 17,000 *g* × 20 minutes); microsome fraction (17,000 *g* × 20 minutes to 100,000 *g* × 60 minutes); and a cell-sap fraction (post 100,000 *g* supernatant). Specific activity (total enzyme released by freeze-thaw expressed per mg. protein) was determined for acid-phenolphthalein-phosphatase and acid-ribonuclease (yeast RNA substrate) for the original homogenate and each of the sub-fractions. Relative specific activity of each fraction (spec. act. fract./spec. act. homog.) was then plotted against per cent of total recovered protein in the fraction. The results for all three species showed the bulk of enzyme activity for both hydrolases to reside in the 17,000 *g* granule fraction with very low relative specific activities in the nuclear and cell-sap fractions. When homogenates were prepared in sucrose containing 0.2% Tergitol-NPX, the bulk of the enzyme activity appeared in the supernatant (cell-sap) and the activity normally present in granules was significantly diminished. This change in enzyme distribution in the presence of non-ionic detergent is characteristic for lysosomes of other tissues.

Localization of acid-phosphatase was also carried out histochemically. Frozen sections of brain tissue briefly fixed in cold calcium-formol (pH 7.0) containing 0.25 *M* sucrose were stained overnight at 37° C. in Gomori's beta-glycerophosphate medium buffered to pH 5.0. Control sections were incubated with substrate-free medium or with complete medium containing 0.01 *M* sodium/fluoride as enzyme inhibitor. All tissue sections were freed of artifact lead phosphate deposits by brief immersion in dilute acetic acid. The results showed granular staining in neurons of the periventricular and intermediate neuropil layers of olfactory and optic lobes in frog and bass brain. Flounder brain failed to react. All control sections were negative.

Supported by U.S.P.H.S. Training grant 1 T1-GM 1030-02.

Structural changes in the mitotic apparatus after isolation. ROBERT E. KANE.

Experiments carried out over the past several years have led to the development of a simple method for the mass isolation of the mitotic apparatus (MA) from echinoderm and other marine eggs. Immediately after isolation the MA dissolves rapidly in 0.6 *M* KCl, but becomes progressively more insoluble with time. The loss of solubility is a function of temperature and occurs in a few hours at room temperature and in 24 to 48 hours at 0° C. The loss of solubility is not accompanied by any structural changes visible at the light microscope level, since MA stored for extended periods appear unchanged in phase contrast. However, recent studies with the polarizing microscope have shown that the birefringence of the isolated MA, which is similar to that of the intracellular MA immediately after isolation, declines during storage. This loss of birefringence is correlated in time with the loss of solubility and displays the same temperature dependence. Electron microscope studies have shown that the loss of birefringence is accompanied by the breakdown of the 200 Å tubules seen in the freshly isolated MA. These filaments lose their continuity during storage, breaking down to a series of fine granules, which remain linearly arranged along the path previously followed by the filament. Since this loss of continuity is below the optical limit of resolution, no change is visible in phase contrast, but the breakdown of the filaments appears to be responsible for the loss of birefringence.

Supported by Public Health Service research grant GM 08626 and research career program award K3 GM 20,229 from the Division of General Medical Sciences.

Some anatomical features of the elasmobranch kidney. RUDOLF T. KEMPTON.

A study of kidney tubules injected with ink has shown that in several species the general configuration of the tubule is similar: a double-loop neck, the first half of which is ciliated; an extremely long and tortuous proximal tubule, with a fine and coarse region; a distal tubule which entwines the neck.

The glomerulus is so highly vascular that reconstruction of the glomerular capillaries is almost impossible. However, the intra-glomerular blood pressure must be low, judging from published values of aortic pressures. This fact, coupled with the enormous tubular length, raises a question as to how adequate filtration is achieved.

Resistance to flow through the tubule is presumably at least partially compensated by the long cilia of the first limb of the neck, and by the very powerful flagella of the proximal tubule. While only a small fraction of the proximal tubule cells possess these, each flagellum is so long that it extends past many cells. There seems to be no part of the proximal tubule whose lumen does not contain active flagella.

These flagella could produce a negative pressure, thus increasing the effective filtration pressure, only if the tubular walls were rigid. The walls do seem to have this property. This becomes apparent when a tubule is punctured with a micropipette because the tubule has no tendency to "ride" on the point; it suffers little displacement, and it is entered much more easily than the amphibian tubule. Further, fixed kidney, dissected under the stereoscopic microscope, shows tubular lumina which are wide open. This is also seen in histological sections, in contrast with other kidneys. Finally, the basement membrane is unusually thick and this may well contribute to the rigidity of the walls. It is suggested that this rigidity may play a role by permitting an increase in the effective filtration pressure.

The secretion of phenol red by the smooth dogfish, Mustelus canis. RUDOLF T. KEMPTON.

Preliminary to the use of puncture methods to determine the site of tubular secretion of phenol red in the elasmobranch (*Narcine brasiliensis*), study has been made of the secretion of this dye by the smooth dogfish. The resulting data, together with a report by Willie Smith on the secretion of phenol red by the spiny dogfish, give assurance that there is sufficient secretion of phenol red to permit its study by puncture methods.

The secreted fraction of the eliminated dye is impressively large when the plasma level is of the order of 6 mgm.% or lower. In 69 collection periods in this concentration range, the lowest fraction was over 50%, the highest 98%, the mean 87%. This secretion, together with water reabsorption, gave urine/plasma concentration ratios of phenol red with a mean value of 19.4, in contrast to the 2.36 mean of the inulin U/P.

Although the data are somewhat scattered, it seems clear that a tubular maximum is reached at plasma levels between 2 and 3 mgm.% This is in close agreement with the 2 mgm.% level found by Willie Smith in the spiny dogfish. Also in agreement between the two genera is the trend of the ratio between the clearances of phenol red and inulin, with high ratios (maximum 68.5, mean 13.2) in the 24 periods in which the plasma levels were below 1 mgm.%.

Differences between the genera include the fact that the rate of filtration (inulin clearance) in the smooth dogfish is only about one third of that of the spiny (33 ml./day/K *vs.* 92.8 ml./day/K) as reported by Smith; and the maximal tubule secretion rate for the smooth dogfish is 5.4 mgm./day/K as contrasted to 18 mgm./day/K for the spiny dogfish. It is not clear whether these differences would disappear if the basis of comparison were kidney mass rather than gross body weight.

Is there long-lived mRNA in diapausing pupae of the Cecropia silkworm? A. KRISHNAKUMARAN AND H. A. SCHNEIDERMAN.

Diapausing pupae of *Hyalophora cecropia* have no DNA synthesis in any tissues except hemocytes. This appears to be the primary biochemical defect preventing their development. This developmental arrest is accompanied by: low respiration rate, defective cytochrome system, low RNA content and rate of RNA synthesis, low rate of protein synthesis. How long can they survive if RNA synthesis is blocked and how long will they continue to make proteins? Autoradiographic studies revealed that 0.25 micrograms per gram of actinomycin D completely blocked nuclear RNA synthesis in all tissues of diapausing pupae. A series of pupae was injected with 40 times this effective dose (*i.e.,* 10 micrograms per gram) every four days, along with streptomycin and penicillin to prevent infection. Half of the pupae were still alive on the 24th day after they each had received 6 injections totalling 60 micrograms per gram of actinomycin. They were then injected with C^{14}-labelled amino acids and the ability of several tissues to synthesize proteins examined. Fat body synthesized protein at less than 3% the rate of normal diapausing pupae. By contrast, proteins in the blood were well labelled, indicating that protein synthesis continued in certain tissues even after 24 days of treatment with actinomycin. The significance of these findings in relation to possible long-lived mRNA will be discussed. Appropriate RNA–DNA hybridization experiments are presently being conducted to determine (a) whether labeled RNA, which can hybridize with DNA, and which was synthesized before actinomycin treatment, persists in 24-day-old actinomycin-treated pupae, and (b) how completely actinomycin blocks the incorporation of labeled uridine into various RNA fractions.

This research was supported by an A.I.H. grant. A. Krishnakumaran was supported by an N.I.H. postdoctoral traineeship in developmental biology.

Preliminary studies on dogfish corneal DNA. SIDNEY LERMAN, GEORGE MUNRO AND SEYMOUR ZIGMAN.

The dogfish corneal epithelium represents almost one half the mass of the entire cornea and is rich in DNA (460 micrograms per 100 mgm. dry weight.) The RNA:DNA ratio is approximately 2:1.

Purified DNA was prepared by the detergent extraction of nucleic acids from the 600 *g* sediment of the corneal homogenate. The RNA was hydrolyzed in 0.3 N KOH for 18 hours at 37° C. and the mixture of nucleotides and DNA was separated on a G-200 Sephadex column (7 cm. × 0.5 cm.) in 0.025 *M* Tris pH 7.4. The DNA was totally eluted in 4 ml. and the remaining ribonucleotides came off in the next 5 ml. A 98% recovery of DNA was obtained with this method. The DNA thus obtained had a 258/280 absorption ratio of 2, a sedimentation constant of 46s and had a molecular weight of approximately 2×10^6.

Metabolic studies on this corneal DNA revealed that when 10^{-7} *M* tritiated thymidine was added to the incubation media, it was incorporated at a rate of 1.4×10^{-5} micromoles per hour. When corneal epithelium was incubated in the cold (0° C.) or with nitrogen substituting for oxygen in the gas mixture, the rate of thymidine incorporation was depressed by approximately 50%. IUDR was not incorporated into DNA when it was added to the incubate at 10^{-8} *M*, but when the concentration of IUDR was increased 10-fold it was incorporated into DNA at an appreciable rate. It required a 10-fold excess in the concentration of cold IUDR, as compared to H^3 thymidine, to achieve a significant inhibition of thymidine incorporation (approximately

40%). A similar degree of inhibition of H^3 thymidine incorporation could be obtained by adding cold thymidine in a concentration equivalent to the amount of IUDR added.

Supported by Fight For Sight Fellowship, National Council to Combat Blindness (F-1692) and A.E.C. and O.N.R. grants (M.B.L.).

Mustelus canis pepsinogen and its reaction with rabbit anti-pepsinogen. LAWRENCE LEVINE AND HELEN VAN VUNAKIS.

Pepsinogen, extracted from the stomach mucosa of *Mustelus canis,* has been purified by ammonium sulfate precipitation and DEAE cellulose chromatography. The purified pepsinogen was ultracentrifugally homogeneous. Antisera to the purified pepsinogen were prepared in rabbits. In gel diffusion one major and one minor precipitating band developed when crude mucosal extract was used as the diffusing antigen. Only the major band was observed when the purified pepsinogen was used as the diffusing antigen. The rabbit antisera to *Mustelus canis* pepsinogen were used to examine the relationships of pepsinogen and pepsin from other cartilaginous fishes. Pepsin, generated from the purified pepsinogen under acid conditions, reacted with the anti-pepsinogen, but required $2\frac{1}{2}$ times more antibody. The pepsinogens from the sand shark (*Carcharias taurus*), dusky shark (*Carcharhinus obscurus*), and the blue shark (*Prionace glauca*) all reacted with the anti-pepsinogen but required 4, 6 and 6 times more antibody, respectively, to give the equivalent fixation obtained with the smooth dogfish pepsinogen. The Atlantic sting ray (*Dasyatis sabina*) also reacted but needed 50 times more antibody.

The rabbit anti-pepsinogen was examined for its stability to various denaturing agents. It was found that the antibody begins to denature after incubation at 67° C. for 30 minutes with 50% denaturation obtained after 30-minute incubation at 73° C. The effects of the amide and alcohol series of denaturing agents were examined by incubation of the antiserum for 30 minutes at 60° C. in the presence of varying concentrations of the denaturing agents. The concentrations of the amides giving 50% denaturation under these conditions were formamide (4.1 M), acetamide (2.3 M), propionamide (1.4 M), butyramide (0.75 M), and hexanamide (0.14 M). The concentrations of the alcohols giving 50% denaturation under these conditions were methanol (3.4 M), ethanol (1.65 M), n-propanol (0.76 M), and n-butanol (0.32 M). It was concluded that hydrophobic forces stabilize the antibody receptor site.

Supported by research grants from the NIH (AI 01940 and AI 02792).

Inhibitors of fibrin polymerization. L. LORAND, A. JACOBSEN AND R. SCHUEL.

In 1962 we described the crosslinking of vertebrate fibrin (*i.e.,* transformation from a 1% monochloroacetic acid-soluble gel to an insoluble one under the influence of the thrombin-activated fibrin-stabilizing factor: FSF*) as a reaction between amino ("donor") groups of one fibrin molecule and carbonyl ("acceptor") functions of another. Such a transpeptidation (*i.e.,* transamidation) mechanism predicts two types of simple inhibitors. Amines could compete with the "donor," and carbonyl derivatives with the "acceptor" functions of fibrin. We proved the existence of both inhibitors, exemplified by glycine ethylester and N-carbobenzoxy-L-aspartic acid diamide, respectively, and demonstrated the incorporation of the former into fibrin by FSF*. These inhibitors are significant because they (1) enable one to label specifically the polymerizing centers of the fibrin molecule; (2) permit the development of synthetic substrates for FSF*; (3) represent a novel group of inhibitors of vertebrate blood coagulation which do not interfere with gelation of fibrin but only delay crosslinking of the gel, rendering it more susceptible to fibrinolytic enzymes; and (4) signify the possibility of the fibrinogen-fibrin-FSF* system acting as a mechanism for the incorporation (detoxification) of amines in general.

The clotting time of *Homarus americanus* plasma is considerably prolonged by amines which inhibit the crosslinking of vertebrate fibrin. In addition to the compounds already described, histamine, serotonin, and 2,5 d-aminoxyalaninediketopiperazine were found to be strongly inhibitory (<1 mM). Tryptamine, ϵ-aminocaproic acid, aminomethanesulfonic acid, and ammonium acetate were mild inhibitors (<20 mM).

Aided by U.S. Public Health Service Research Career Program Award HE K6 3512 and by grant HE 02212 from the National Heart Institute.

Partial reversal by high pressure of the anti-meiotic effects of heavy water in the öocytes of the starfish, Asterias forbesi. Douglas Marsland[1] and Yukio Hiramoto.[2]

Although it is well known that mitotic division can be blocked by replacing 70% or more of the H_2O content of the system with D_2O, there is very little information as to the effects of deuteration on meiotic division. In this area, the starfish provides a favorable material, since the first meiotic division is initiated soon after the öocytes are liberated into sea water and the formation of the second polar body is complete within about two hours at 20° C., if the öocytes are inseminated within the first hour.

As previously reported, the antimitotic effects of deuteration in the range of 70–90% D_2O can be partially reversed in cleaving echinoderm eggs by high pressure in the range of 4000–5000 psi. Consequently, it became of interest to investigate these effects with reference to the maturation divisions of the starfish öocyte.

A partial reversal of meiotic blockage was found in two types of experiment. In the first, the deuteration and pressure treatments were initiated early, about 5 minutes before the breakdown of the germinal vesicles, and a complete disappearance of the vesicles was taken as a criterion of meiotic progress. In the second, the treatments were started later, some 5–10 minutes before the expected appearance of the first polar bodies, and the formation and persistence of the polar bodies provided the criterion. In all of the experiments, the temperature was fixed at 20 ± 0.5° C.

Deuteration alone, in the range of 60–80% D_2O in sea water, blocked maturation, as judged by the fact that the breakdown of germinal vesicles was not complete. With pressure, however, a significantly high percentage of complete breakdown was observed. Specifically, the maximal breakdown percentages were: 90% at 3500 psi, for 60% D_2O-sea water; 40% at 4000–5000 psi, for 70% D_2O and 25% at 5000–5500 psi, for 80% D_2O.

Similarly, polar body performance was considerably improved by pressurization, particularly with reference to persistence after formation. Specifically, the maxima of persistence were observed to be 77%, 55% and 65%, respectively, for öocytes in 70%, 80% and 90% D_2O-sea water, when pressures in the range of 2500–3500 psi were applied, whereas in the absence of applied pressure the persistence was less than 2%.

Succinic dehydrogenase activity in Tubularian development. James A. Miller, El Sayed Hegab and Faith S. Miller.

Succinic dehydrogenase activity was studied in living embryos of *Tubularia,* in fresh frozen and in prefixed whole-mounts and sections. In most of the studies a modification of the method of Nachlas *et al.* was used. Controls incubated without substrate or with malate showed no activity.

In general, succinic dehydrogenase activity is low in early stages and increases during development. Regional differences appear even in the uncleaved egg, with greater activity along the future aboral surface than the oral and the greatest activity at the junction of the two where cleavages appear later.

The first evidence of tentacle formation is increased succinic dehydrogenase activity in the endoderm. This is followed shortly by a great increase in activity of the ectoderm around the base of the tentacles. The former center of activity gradually fades out but the base of the tentacle maintains its activity throughout the developmental period.

The activity of the aboral surface increases during development and becomes localized into two areas, the holdfast region at the apex and the subterminal perisarc-secreting zone. In the late actinula this is the most active region in the entire larva.

The development of the mouth and distal tentacles is accompanied by increases in succinic dehydrogenase activity in the endoderm but not in the ectoderm. Distal to the bases of the proximal tentacles areas of activation develop which involve both ectoderm and endoderm. These are the developing gonophores. The highly active endoderm of these gives rise to the germinal cells of the gonophore.

---

[1] Work supported by grant series CA 00807, from the National Cancer Institute, U.S.P.H.S.

[2] Lalor Fellow at M.B.L., 1964.

Thus, each step in the development of *Tubularia* is accompanied by changes in succinic dehydrogenase activity. Since in several cases the activity change precedes visible structural changes, the results do not confirm the concept that structure determines function. Actually, these results are consistent with the concept that function precedes and determines structure.

Isolation of the chemotactant of Campanularia (Laomedea) calceolifera. RICHARD L. MILLER.

Large elliptical aggregations of motile sperm seen downcurrent from the female gonangium of *Campanularia* (*Laomedea*) *calceolifera* appeared to be the result of the drift of a stable factor. Insertion of a micropipette with tip diameter of 100 μ into the opening of the gonangium and slow suction by means of a hand-controlled micrometer syringe yielded 0.1 to 0.5 microliter of sea water with attracting activity. If injected into a suspension of *C. calceolifera* sperm, an increase in motility of sperm near the tip of the pipette was seen, followed by an aggregation. Control sea water injections had no effect. The sperm respond to the factor almost exactly as they do to the female gonangium. Attempts to isolate the chemotactant of *C. flexuosa* by the same method have led to equivocal results. A few clearly positive cases have been seen, however.

If a small number of female *C. calceolifera* gonangia are placed in 2–3 ml. of filtered sea water with or without streptomycin (20 mg./ml.) and penicillin (200 units/ml.) for one or more hours, the chemotactant may be obtained in greater quantity. Titer of the solutions so obtained, as defined by the highest dilution showing sperm activation, ranges from 32 to more than 512, depending upon the length of extraction and the state of the gonangia. The factor does not attract or activate *C. flexuosa* sperm, is stable in the cold for at least five days, is not affected appreciably by boiling for 30 minutes and is unaffected by freezing. Preliminary observations show little or no UV absorption at 230, 260 or 280 millimicra.

Supported by U.S.P.H.S. Grant 5 T1 GM 150-04.

Extraction of ATPase from sea urchin and fish sperm tails. HIDEO MOHRI.

Proteins which may be involved in the movement of flagella or cilia are hardly extractable with salt solution at neutral pH. In the present experiment, Gibbons' method (1963), which was reported to extract most of the ATPase activity from *Tetrahymena* cilia, was applied to sea urchin (*Arbacia punctulata*) and fish (*Prionotus carolinus*) sperm tails.

Spermatozoa were suspended in a Ca- and Mg-free artificial sea water and homogenized in a glass homogenizer. Tails were separated from heads by centrifugation at 1000 *g* for 10 minutes and sedimented from the supernatant at 10,000 *g* for 10 minutes. The sedimented tails were extracted twice with 0.5% digitonin solution and then subjected either to overnight extraction in Weber-Edsall solution or overnight dialysis against a Tris-EDTA solution. In the case of *Arbacia* sperm, both treatments extracted more than 80% of ATPase activity remaining in the digitonin-treated tails. The extracted ATPase split only the terminal phosphate of ATP, and had a Q_p value of about 500 at 21° C. (more than 1000 at 37° C.), about twice as high as that of freshly isolated tails. The enzyme activity was stimulated by both Mg and Ca, the former being more effective. A pH optimum was found around 9.0. In the case of *Prionotus* sperm, however, the same treatments solubilized only half of the ATPase activity, even after the digitonin extraction. The solutions containing active ATPase showed an absorption maximum at 278 mμ and an absorbancy ratio 280/260 mμ of about 1.3. A 5% PCA extract of the protein had a maximum at 258 mμ, indicating the presence of nucleotide. Some efforts have been made to characterize the extracted proteins.

Supported by a faculty summer research award from the Lalor Foundation.

In vitro studies on the shedding activity found in Asterias nerve extracts. B. DIANNE MOORE AND J. D. BIGGERS.

An *in vitro* assay system has been developed for testing the shedding activity in *Asterias* nerve extracts and other substances. The ovaries were removed, leaving the ducts intact, weighed, and placed individually in 70 ml. of sea water. The 10 ovaries from each starfish formed one replicate of a randomized block experimental design. The material to be assayed was added to the surrounding medium and the ovaries reweighed $\frac{1}{2}$–1 hour after the shedding of mature eggs began. The results were analyzed by analyses of variance and covariance, eliminating variation in initial ovarian weight. The higher the dose of extract, the more eggs were shed, and the greater the loss of weight by the gonad. A linear dose-response line was obtained between doses equivalent to 10^{-2} and 10^{-1} of a nerve. Several preparations showed this relationship: (a) the supernatant from fresh or frozen nerves extracted in distilled water for one hour, (b) water extracts of acetone-dried nerves, and (c) lyophilized water extracts of acetone-dried material. Noumura and Kanatani (1961) reported that an acetic acid extract of radial nerves was active *in vivo*. However, a 0.25% acetic acid extract was found to be approximately three times less active *in vitro*.

No marked difference in activity was observed between extracts of nerves from ripe males, females, and from animals that had already shed their gametes.

The *in vitro* shedding activity of purified mammalian pituitary hormones was tested. Oxytocin, vasopressin, LH, FSH, as well as acetylcholine, did not cause shedding. LH and FSH were reported inactive *in vivo* by Chaet (1961). Cysteine, which splits disulfide bonds, inhibited the nerve extract activity at concentrations of 10^{-2} *M* and greater. Such inhibition is characteristic for oxytocin and vasopressin and median eminence-releasing factors which contain a cystine molecule. There is thus a possibility that the active material in the nerve extract may be a neurosecretion similar to those found in higher invertebrate and vertebrate forms.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

Selective block of Na-activation in voltage-clamped squid giant axon and eel electroplaque by tetrodotoxin. Y. NAKAMURA, S. NAKAJIMA AND H. GRUNDFEST.

Tetrodotoxin (puffer-fish poison) blocks spike electrogenesis in various cells. In frog muscle fibers and lobster axons the block is caused by selective elimination of Na-activation. Although different patterns of ionic processes occur in the spike electrogenesis of squid giant axons and eel electroplaques, voltage clamp data show that in these cells, also, block of spikes is effected in the same way.

Clamping current for the axons was delivered through an axial intracellular Ag-AgCl electrode. A Ag-AgCl probe electrode in the center of the voltage-clamped region sensed the membrane potential. Prior to applying tetrodotoxin the ionic currents were of the usual form for this cell. An initial brief inward current, due to Na-activation, was followed by an outward current which resulted from the later K-activation. The initial inward component, and it alone, was eliminated by applying tetrodotoxin, 10^{-8} to 10^{-6} w/v. The effect of the drug could be reversed.

The large currents required to voltage-clamp the electroplaques were applied through large external electrodes. Microelectrodes which straddled the caudal surface sensed the potential of the reactive membrane. Only the initial inward current and increase in conductance due to Na-activation occur during spike electrogenesis of eel electroplaques. K-activation is absent. Instead, the conductance of the reactive membrane decreases two- to four-fold from the resting value an indication of depolarizing K-inactivation. Tetrodotoxin eliminated the inward current but not the decrease in conductance.

Thus, processes of K-inactivation as well as of K-activation are not affected by the poison, which acts specifically to cause pharmacological Na-inactivation and only in the electrically excitable electrogenic membranes in phylogenetically diverse tissues. The pharmacological specificity indicates that electrically excitable membrane is heterogeneous, changes in permeability for Na and K occurring at sites which have different chemical structures.

Supported by grants from NIH (B 3728, NB 03270-02 and 2B 5328 (R1)) and NSF (G-19969) to H. Grundfest.

Mechanics of toadfish swimbladder muscle. MOON JAE PAK AND BERNARD C. ABBOTT.

Small bundles of muscle fibers with motor nerve have been isolated from toadfish swimbladder. Attachments were to tendon at one end and to a piece of swimbladder at the other.

Stimulation was through nerve or through massive electrodes. With suitable bathing solution, isolated fibers lived for several days. Isometric, isotonic contractions and electrical responses were studied.

Resting membrane potential at 20° C. averaged 74.2 mv. and action potential 88 mv. Tension appeared during the course of action potential. Fused tetani were not obtainable. Peak twitch tension of 41 gm./cm.^{2} was maximal at 6° C. Time to half relaxation at 20° C. was 3 msec. and increased steadily to 0° C. with Q_{10} of 4; but rate of tension rise increased as temperature fell to peak value at 6° C. This suggests (as does the lack of fusion at 20° C.) that relaxation events begin so early that they interfere with activation processes.

Decay of active state was measured by time to maximum shortening in isotonic contractions at increasing afterloads. At 6° C. the decay took 50 msec. (and tension falls at almost as rapidly) and corresponds at 20° C. to complete decay of active state in 6 msec. from peak of tension. At 4° C. the maximum speed of isotonic shortening is 1.5 M. length/sec. so it is not intrinsically a fast muscle and the very brief twitches are due to extremely fast relaxation processes.

The contractile material forms the cortex of the 10–15 μ fiber around a central core of sarcoplasm containing mitochondria, and the fibers are radial wedges 0.3 μ wide separated by sarcoplasmic reticulum. The distance from all contractile material to reticulum is very small and probably relates to the rapidity of onset and decay of activation.

This work was supported by the Comparative Physiology Training Program U.S.P.H.S. GM 1030.

The permeability of the plasma membrane of the egg of Arbacia punctulata does not alter on fertilization. ARTHUR K. PARPART.

It has long been known that activation, by means of sperm or by many parthenogenic agents, of the eggs of *Arbacia punctulata* leads to an increase in permeability of these egg cells to a variety of compounds. In all cases previously studied, the activating agents have caused the explosive breakdown of the cortical granule layer that lies between the plasma membrane of the egg and the outermost vitelline membrane; also, these agents induce nuclear division and subsequent cell division in the fully activated egg.

Electron micrographs show the cortical granule layer to be an exceptionally electron-dense region which decreases greatly on activation. Also, this region does not show distortion under high centrifugal force. A solution to this aspect of the problem could be activation of the egg for nuclear division and cleavage without breakdown of the cortical layer.

This latter solution has been obtained by exposing *Arbacia* eggs to a mixture of 0.005 M cysteine-HCl + 0.1% egg albumin dissolved in sea water and brought to pH 3.80. Exposure of the eggs to this mixture for 20 minutes, followed by four subsequent washings with sea water, produces activation of over 90% of the eggs without breakdown of the cortical granules.

Comparison of the penetration of ethylene and propylene glycols shows these cysteine-acid-activated eggs to have the same rate of penetration as with the unactivated egg. However, if the cortical granules are broken down by the usual parthenogenic agents after cysteine activation, the rate of penetration of these compounds increases to that of the fully activated egg.

It would appear from the foregoing type of experimental result that the permeability of the plasma membrane of the egg of *Arbacia punctulata* is not altered in the fully activated egg as has previously been claimed. Rather, the difference in permeability between unactivated and fully activated eggs is to be associated with a diffusion delay within the cortical layer of the unactivated egg.

Separation of Griffithsia globulifera DNase from phycoerythrin. JOHN PAWELEK AND MAIMON NASATIR.

An acetone powder was prepared from the red alga, *Griffithsia globulifera*. An extract was made from the powder in a 0.02 M PO_4, 0.001 M citrate buffer (pH 6.93). After centrifugation at 32,000 g at 2° C., the pink supernatant fraction was tested for DNase activity, using C^{14}-labeled *E.* coli DNA mixed with non-radioactive calf thymus DNA as a substrate. Approximately 35% of the radioactive substrate was degraded in two hours at 25° C.

An attempt was made to separate the pink algal protein, phycoerythrin, from the DNase activity. An extract prepared as above was placed in dialysis bags and concentrated for 6–8 hours with Aquaside. The concentrated protein extract was placed on a Sephadex G-200 column and eluted with 0.1 *M* NaCl at 2° C. Two major fractions were recovered: a pink fraction in the first 21 ml. and a colorless fraction absorbing at 280 mμ in the 25th to 29th ml. The colorless fraction was assayed for DNase activity using the same substrate. Approximately 70% of the radioactive substrate was degraded in the first 10 minutes.

This work was in part supported by a grant from the National Science Foundation, NSF GB-36, and by a training grant from the National Institutes of Health, 8T1 19-04.

On reproduction in Pinnotheres maculatus (Decapoda: Pinnotheridae). Jack B. Pearce.

From 5 July 1963 to 5 August 1964, 97.6% of 1820 *Mytilus edulis* collected from an epibenthic mussel bed at Quicks Hole (41°26′15″N, 70°50′50″W) were infested with the parasitic mussel crab, *Pinnotheres maculatus.* Ovigerous females were first noted in late May. By mid-June of 1963 and 1964 almost all mature females carried eggs. These began to hatch in early August. Zoeal and megalopal stages are planktonic. In mid-September, following the planktonic larval stages, the megalops molted into a true first crab which soon left the plankton and became associated with a bivalve host, usually benthic *Mytilus edulis* in the Woods Hole area.

Several molts occurred, each succeeding instar being somewhat larger but morphologically similar to the preceding crab stages. By mid-October both male and female crabs reached an average carapace width of 3.3 mm. At this time both sexes molted into an anomalous juvenile instar. Unlike previous instars this stage had a well-calcified hard exoskeleton and other adaptations for a free swimming planktonic existence. During this stage females and males left their host and engaged in *copulatory swarming* in open water. Sex ratio of swarming crabs was 1:1.

Following copulatory swarming females settled from the plankton and again infested a bivalve. After a host was entered, four posthard molts occurred. Each led to a well-defined instar having unique characteristics: soft, poorly calcified exoskeletons adapted to a symbiotic existence. Juvenile females, which entered hosts already inhabited by mature females, were retarded in development and did not reach the sexually mature Stage V instar. All crabs apparently overwintered in the first, *posthard* instar (Stage II). Male crabs spent a greater length of time in open water during copulatory swarming and hence were more subject to predation. Few males were found in hosts following swarming.

With the advent of higher water temperatures in May the precociously inseminated crabs passed through the remaining three *posthard* molts to the adult instar and some became ovigerous at the end of their first year.

Copulatory swarming behavior has been noted in only one other pinnotherid, the West Coast mussel crab *Fabia subquadrata.* Inhibition of development in juvenile crabs due to the presence of the adult female has not been previously observed in the Pinnotheridae.

Supported by Grant GB-561 from the National Science Foundation.

Some preliminary results of a taxonomic study of the Hydrozoa of the Cape Cod area. K. W. Petersen.

The West-Atlantic boreal region has been subdivided into three zoogeographic provinces: the Newfoundland-Nova Scotia, the Gulf of Maine, and the Cape Cod-Cape Hatteras. Cape Cod is regarded as the boundary between the latter two provinces. Out of 25 species of Hydromedusae occurring in the Gulf of Maine, only 7 also occur in the southermost province, while 18 also occur in the East-Atlantic boreal region. Out of 32 species in the Cape Cod-Cape Hatteras province, only 10 also occur in the East-Atlantic boreal and the Mediterranean.

To most species belonging to the southern province, Cape Cod constitutes an absolute barrier, while several of the northern species from the Gulf of Maine province penetrate for some distance to the south of Cape Cod, occurring both in Buzzads Bay and Vineyard Sound. These species reproduce during the spring, where the temperatures are almost the same in Cape Cod Bay and Vineyard Sound. There is, however, a difference in the reproductive periods of these species south and north of the Cape. For example *Sarsia eximia* (Allman) (which is here recorded for the first time from the Atlantic coast of North America) reproduces south of the Cape in March, north of the Cape in April–May. For *Hybocodon prolifer* L. Agassiz, the corresponding periods are March–April and July, and for *Rathkea octopunctata* (M. Sars) March–April and June–July. Although temperatures during winter and spring in Vineyard Sound and Cape Cod Bay correspond rather closely to those in Kattegat in the European boreal region, the reproductive period of the amphiatlantic species reproducing in the spring tends to fall one to two months earlier at Cape Cod than in the Kattegat; this may be connected with the greater turbulence and lack of thermal stratification around the Cape, resulting in an early phytoplancton maximum.

Supported by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

Direct continuity of sarcolemma with Z-band of Limulus heart muscle. DELBERT E. PHILPOTT.

Previous work (de Villafranca and Philpott) with *Limulus polyphemus* striated muscle revealed tubular components in the contractile elements, which were believed to be connected with the sarcolemma. I now report that electron microscope study of *Limulus* heart muscle reveals invaginations of the sarcolemma which traverse the cytoplasm and establish direct contact with the Z-band of the muscle. The unit membrane of the sarcolemma is seen to be continuous throughout this structure. Smaller secondary channels or tubules branch off from the main channel and are seen to project into the muscle substance. Several of the invaginations exhibited structural specialization, in that instead of direct contact between membrane and Z-band, a dark area is seen to intervene between membrane and Z-band. The implication of the above described system is that it presents a means by which it is possible to effect a decrease in the time required for the wave of depolarization to activate the contractile system. The wave is no longer required to traverse the cytoplasm, since it is brought directly to the Z-band.

Studies on the infection of the lobster, Homarus americanus, with Gaffkya homari. HARVEY RABIN AND FREDERIK B. BANG.

Gaffkya homari causes a fatal disease of lobsters. Thirty-two per cent of lobsters obtained from M. B. L. Supply in 1963 and 14% in 1964 were positive for *Gaffkyo*-like organisms. Ten per cent of lobsters obtained from a lobsterman in Woods Hole were positive. No *Gaffkya*-isolations were made from lobsters from either the Massachusetts lobster hatchery or from a dealer on Martha's Vineyard.

The effects of *Gaffkya* and a marine *Vibrio* on lobsters were compared. Six animals were inoculated with *Gaffkya* and six with *Vibrio,* and then the twelve were housed together. *Gaffkya* was isolated from all *Gaffkya*-inoculated animals and all died one to nine days after inoculation. No successful isolations of *Vibrio* were made but the *Vibrio*-inoculated animals became infected with *Gaffkya* and died between the fourth and tenth day.

Following inoculation with 5×10^5 *Gaffkya,* lobsters became bacteremic on the first day after inoculation, developed progressively increasing counts, and died on the third and fourth days. Inoculation of *Vibrio* endotoxin 10 hours prior to *Gaffkya* inoculation did not influence the course of the infection. Animals inoculated with endotoxin alone but housed with infected lobsters remained free from *Gaffkya,* while one out of three untreated animals developed a slight *Gaffkya* infection but survived while the other two remained free from *Gaffkya.*

The inoculation of endotoxin caused a prompt but temporary depression in the amebocyte count. Six out of seven lobsters inoculated with *Gaffkya* 12 minutes after receiving endotoxin died within one day.

Lobster serum was tested for its effect on the growth of *Vibrio* and *Gaffkya in vitro.* Sera from ten *Gaffkya*-free lobsters increased the growth of *Gaffkya* 2.2- to 7.5-fold compared with broth controls but had no effect on *Vibrio.* Sera from two animals, which had had slight infections with a *Gaffkya*-like organism, increased the growth of *Gaffkya* but inhibited the growth of *Vibrio* 5- to 8-fold.

Supported by a training grant from the National Cancer Institute.

Delay of cell division in Paramecium aurelia induced by fluorophenylalanine. Leif Rasmussen.

Delays of division in *Paramecium* caused by noxious agents of various biochemical specificities, applied at different times in the cell generation, have been investigated systematically. Results obtained with DL-p-fluorophenylalanine are reported here.

If single cells were *continuously* exposed to 8 m*M* fluorophenylalanine at any time before the completion of 80% of the cell generation (which was 5.5–6.5 hours long), they failed to divide. If they were exposed during the final 20% of the cell generation they divided once. *Short-duration* exposures (one-hour) to 16 m*M* fluorophenylalanine at different times in the cell generation (followed by washing in L-phenylalanine) caused division delays with lengths varying with the time of application of the inhibitor. Prior to completion of 80% of the cell generation, the amount of delay was cell age-dependent; the shortest delay (one-half hour) was obtained with the youngest (post-cleavage) cells and the longest delay (3½ hours) with the cells which were 80% of the way through their cell generation. The time of completion of 80% of the cell generation is called the transition point. Cells treated after the transition point were only delayed an average of 30 minutes, but the length of the next cell generation (the second generation) was extended an average of 90 minutes. Cells treated prior to the transition point did not have a lengthened second generation.

Since fluorophenylalanine sensitivity decreased sharply at the transition point in a cell generation, and cells treated after the transition point were delayed in the next cell generation, the inhibitor must act on processes essential for preparation for division, which are completed at the transition point, and not directly on processes concerned with the division itself.

The transition point in logarithmically-multiplying *Paramecium* (80% of a cell generation) and *Tetrahymena* (65% of a cell generation) coincides with the time of completion of DNA-synthesis. It remains to be seen if this coincidence has a cause-and-effect basis or is fortuitous.

Some effects of salinity, temperature and photoperiodism on the growth and morphogenesis of Ulva lactuca. Ina Katherine Rea.

Zoospores of *Ulva* were grown in Erdschreiber's medium at salinities of 5, 10, 25, 32 and 45‰. Two salinity series were placed in continuous light, one at 19° C., one at 11° C.; another was in a 16-hour light, 8-hour dark cycle at 19° C. The zoospores at 11° C. failed to germinate. At the end of one week, in both continuous light and periodic light, the plants growing in a salinity of 5‰ had the longest filaments and rhizoids with the most lobes. Plants growing at other salinities had short filaments and few, blunt rhizoid lobes. Plants growing at 10‰ showed deformities such as acute twisting and bulging of filaments. After two weeks plants growing in 32‰ had filaments four times as long as those growing at 5‰, about three times as long as at 25‰, four times as long as at 10‰, and 1½ times as long as at 45‰. Plants grown in cyclic light were considerably larger than those in continuous light. In a salinity of 32‰, filaments in the cycle were seven times as long as those in continuous light; in 25‰ six times; in 10‰ five times; in 5‰ two times, and in 45‰ ten times. Further experiments have been set up under different photoperiods.

Supported by NIH Training Grant 5T1 GM 535-04.

Amino acid incorporation by isolated mitochondrial fractions of unfertilized Arbacia eggs and late gastrula embryos. Minocher C. Reporter.

While the incorporation of amino acids into mitochondrial protein is energy-dependent, it does not seem to require the mediation of ATP, in the light of Bronk's (1963) report that the rat liver mitochondrial system was largely insensitive to inhibitors of ATP synthesis. A comparison was made of the ability of mitochondrial fractions of unfertilized *Arbacia* eggs and gastrula embryos to incorporate amino acids under the influence of oligomycin, in order to test for the presence of an ATP-independent mechanism in embryonic systems. Electron microscopic examination was used to check the purity of mitochondrial preparations. The initial 12,000 *g* pellets were recentrifuged in discontinuous density gradients of sucrose-Tris solutions. Mitochondria were collected at the interface between 1.00 and 1.23 *M* sucrose for unfertilized eggs, while for the embryos they were collected at the interface between 1.00 and 1.22 *M* sucrose. The final pellets of mitochondrial fractions were suspended in a medium of sucrose 0.3 *M*; Tris 0.1 *M*, pH 7.2; EDTA 10^{-5} *M* and KCl 0.3 *M*. Incorporation experiments were performed, using 0.25 micromole of each of 18 amino acids; C^{14}-valine; NAD^{+} and Mg^{++} with beta-hydroxybutyrate as substrate. In the presence of 0.1 microgram/ml. oligomycin, the linear rate of incorporation of amino acids between 5 and 30 minutes into mitochondrial protein of unfertilized eggs was 5440 cpm per mg. protein per minute, as against 2560 cpm for control homogenates. The respective figures for embryos were 1390 cpm and 8.89 cpm. Other experiments confirmed such differences and also showed inhibition of incorporation by ADP and antimycin A. An experiment with thyroxine (10^{-6} *M*) caused a three-fold increase in incorporation at the end of 30 minutes in embryonic, as contrasted to its relatively small effect on unfertilized egg, mitochondria. Incorporation of amino acids thus indicates that mitochondria of unfertilized eggs have a larger capacity to furnish high energy intermediates than mitochondria from gastrula embryos.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

Unusual deoxyribonucleic acids isolated from the sand dollar, Echinarachnius parma. HERBERT S. ROSENKRANZ AND GEORGE A. CARDEN III.

The technique of cesium chloride density gradient centrifugation was used to characterize the deoxyribonucleic acids from haploid and somatic cells of *Echinarachnius parma.* The DNA isolated from the sperm of this species exhibited a buoyant density of 1.700 g./cm.^{3} which corresponds to a guanine-cytosine content of 41%. Upon heating (100° C.) and quick cooling, 99% of the DNA still had a density of 1.700 g./cm.^{3}, while only 1% of the material exhibited the expected shift to a density of 1.714 g./cm.^{3}. If, prior to heating, the DNA was exposed for a short time to sonic vibrations, approximately 70% of the DNA had a density of 1.714 g./cm.^{3}, while about 30% retained a density of 1.700 g./cm.^{3}. These properties indicate that the DNA of the sperm of *Echinarchnius parma* is double-stranded and circular in shape. The DNA isolated from the somatic cells of this organism gave two bands in CsCl, one (~85%) with a density of 1.700 g./cm.^{3} (41% guanine-cytosine), and the other (~15%) with a density of 1.682 g./cm.^{3}. If this second band is indeed composed of DNA, then its density would indicate a guanine-cytosine content of 0.2%; in other words, it is almost a pure copolymer of adenine and thymine. The properties of this unusual component and its developmental origin are currently under investigation.

This study was supported by the Office of Naval Research (Nonr 266-89) and by the U.S.P.H.S. (AI 05111).

Absorption and transport of D-glucose in the intestine of Thyone briareus. CHARLOTTE RUNDLES AND A. FARMANFARMAIAN.

In the first two decades of this century several investigators reported that the digestive tube of holothurians is impermeable to a variety of sugars, amino acids, dyes, and inorganic salts. These reports lead to the view that digested material is translocated by phagocytic ameboycytes from the lumen of the gut to the tissues of the animal. Recent investigations of nutrient transport in the echinoids and asteroids indicate that sugars and amino acids are absorbed and transported across the gut wall and into the perivisceral fluid in the dissolved state. One of us (Farmanfarmaian) has also investigated the transport of D-glucose in the digestive tract of *Leptosynapta* and *Holothuria.* This sugar was observed to cross the gut wall in *in vitro* preparations of intestinal segments from these holothurians.

In the current study of this problem segments of the intestine of *Thyone briareus* were used in a similar manner to see if the various divisions of this gut are permeable to D-glucose, and possibly to clarify the transport mechanism involved. When 0.1 *M* isotonic glucose solution in sea water was placed on the mucosal side and filtered sea water on the serosal side, glucose was detected in appreciable amounts on the serosal side. This was true for all the three divisions of the digestive tract of the organism. The rate of passage varied somewhat from animal to animal but on the average was about 6 μg./hr./mg. dry tissue for the first division; 7.8 for the second division; and about 7 for the third division near the cloaca. These rates were determined at 22° C. plus or minus one degree. This transport of glucose is inhibited by phloridzin, which indicates that simple diffusion is not the mechanism. Preliminary experiments with 4 mM glucose solution, both on the mucosal and serosal sides, indicate that glucose may be translocated by active transport.

Nuclear-cytoplasmic relations in radiation sensitivity. RONALD C. RUSTAD, SHUHEI YUYAMA AND LYNNE C. RUSTAD.

Arbacia and *Lytechinus* eggs were cut in half and both halves fertilized. The diploid half-cells and the whole cells divided simultaneously; the haploid half-cells (merogones) divided 5 to 10 minutes later.

In all of the following experiments, irradiation was performed before fertilization. Both UV and gamma irradiation delayed the division of nucleated half-cells more than the division of whole cells. Whole eggs and enucleated halves were equally sensitive to UV. The gamma-irradiated enucleated cells showed less mitotic delay after fertilization than whole cells, and the dose dependence curves were qualitatively different.

Cytoplasmic organelles were stratified by centrifugation, and different types of partial cells were prepared by cutting along visible bands. Enucleated cells containing the mitochondrial layer were sensitive to gamma-ray-induced mitotic delay. When the mitochondrial layer was removed, radiation-induced mitotic delay could not be detected in the merogones; however, these cells divided later than uncentrifuged irradiated merogones.

Eggs and half-eggs were fertilized with gamma-irradiated sperm. The division delay was greater in nucleated halves than in whole cells and least in merogones. Continuous exposure to actinomysin-D beginning one hour before fertilization did not significantly increase the mitotic delay in whole cells fertilized with gamma-irradiated sperm. Sperm damage led to the death of merogones during the blastula stage at doses which did not affect the development of nucleated cells. Unirradiated half- and quarter-embryos hatched earlier than whole blastulae, but their later development was slower.

These studies were supported by the U. S. Atomic Energy Commission and the Office of Naval Research.

Chemical and biological characteristics of growth-inhibiting agents from Mercenaria mercenaria extracts. II. SISTER M. ROSARII SCHMEER, O.P.

Preliminary studies on the chemical and biological nature of an extract containing a growth-inhibiting principle from the edible quahog, *Mercenaria mercenaria,* have been reported.

Additional purification on Sephadex G-25 gel columns produces a still partially purified, effective agent that causes regression and inhibition of the Krebs-2 carcinoma in female, 3–4-week-old, Swiss albino mice. A typical result, using many test animals, would give a control mean tumor weight of 2200 mg., while the treated animals would have a mean tumor weight of 750 mg. The animals treated to give such three-fold activity receive 1 unit of growth-inhibitor each day for 7 full days. This unit is equal to 20 mg. of the crude, lyophilized clam body that has been reconstituted for application on the Sephadex columns.

Studies in the preparation of an effective, crude extract, that demonstrates a high degree of anti-growth activity, indicate that the active material is released in distilled water. Distilled water may be added to the whole clam body, plus the fluid inside the valves, before homogenizing the *Mercenaria.* Varying percentages of the water, from less than 1% to 100%, can be used to cause the active principle to enter into the supernatant. The usual methods of preparation, reported earlier, but minus treatment with ammonium sulfate, can be used to produce an effective fraction of growth-inhibitor in our bioassay system using the Krebs-2 carcinoma.

The molecular weight of the active principle has been shown to be somewhat less than 5000. Further purification is under investigation to determine the chemical properties of this agent.

Electron and light microscopy studies, under way at this time, are designed to determine the loci of action of our inhibitor at the cytological level using cytochemical techniques.

This investigation has been partially supported by National Science Foundation Fellowship 73182. I wish to thank Dr. Albert Szent-Györgyi for his interest and assistance in some of this study.

A new concept of the mechanism of cytokinesis. Allan Scott.

According to this concept (1) the entire surface of the cell is viewed as competent to undergo contraction and furrowing during the division phase, (2) the mature aster releases an activator-to-contraction around its entire periphery, (3) active contraction occurs at the periphery of an aster wherever competing stresses over the surface and variations of endoplasmic viscosity are favorable, (4) initial furrowing facilitates subsequent furrowing. The concept explains the pattern of furrowing of *Arbacia punctulata* eggs distorted to disc or ribbon shape by flattening. Disc-shaped eggs in the diaster stage may cleave into four pieces in the first division with the furrows cutting in around the asters. Ribbon-shaped eggs, with the diaster fixed in the center of the long axis of the ribbon, may divide the flattened cell transversely into four pieces by three furrows. The middle furrow bisects the spindle and each of the others cuts away an aster from a large mass of protoplasm at the end of the cell. In eggs flattened to a disc shape with the diaster located eccentrically, the furrow cuts slowly across the cell from one side to the other with a furrow-head leading the way. When the collocation of echinochrome granules is used as an indicator of cortical contraction, it is observed that contraction occurs only in the region of the advancing furrow-head. This observation is taken to indicate that contraction at the furrow-head facilitates contraction in adjacent areas and so the furrow is extended. There is a correspondence between the slow advance of the furrow in these flattened echinoderm eggs and the slow movement of the furrow through the amphibian egg.

Electrically excitable systems of voluntary muscle. F. J. M. Sichel.

Originally these experiments were to investigate the direction of an external electrical field optimal for stimulation of parallel-fibered striated muscle, both in the normally conducting state and under conditions of abolished conduction. In normal muscle a longitudinal current is usually considered more effective; on the other hand, our experience has been that a transverse rather than longitudinal field elicits larger isometric responses in non-conducting muscle.

For most experiments the frog sartorius was used. This muscle can be made non-conducting by a suitable elevation of the K^+ concentration of the Ringer's solution, or by various other agents. Such preparations are stable and continue to give twitch-like responses to appropriate stimuli for long periods. They quickly regain the ability to conduct on return to normal Ringer's solution. The stimuli were rectangular current pulses, usually of 1-msec. duration in recent experiments, although both much longer and much shorter pulses have also been used.

As reported previously, in muscles with suppressed conduction, with external field strengths only slightly above threshold, the tension response was greater for longitudinal current than for equal transverse current. Alternatively, the threshold is lower for longitudinal current than for transverse. Since this is as in conducting muscle, it is concluded that probably the same or a similarly oriented excitable system is involved. In contrast to this behavior near threshold, when the external field strength is sufficient to elicit a considerable twitch, the response to transverse current exceeds the response to comparable longitudinal current. A tentative conclusion is that larger currents affect structures oriented differently than the target of smaller currents. This is supported by recent results at threshold which show both these components.

Supported by a research grant, HE 00336, from the National Heart Institute, U.S.P.H.S.

The localization of acid phosphatase in the eggs of several species of invertebrates. Arlan E. S. Smith.

The localization of acid phosphatase in some marine eggs was examined by using the Gomori reaction in conjunction with electron microscopy. Special attention was given to the cortical region because of the possibility that cortical vesicles may be similar in nature to lysosomes.

Whole eggs, unfertilized and fertilized, of three species, *Spisula solidissima, Echinarachnius parma, Ciona intestinalis,* were fixed in Tris-buffered 6% glutaraldehyde, pH 7.8, with 0.3 M KCl added. After fixation the eggs were washed in 0.5 M KCl for 1 hour and then incubated in the substrate solution (0.02 M beta glycero-phosphate, 0.1 M Tris-succinate, pH 5.0, 0.0024 M $Pb(NO_3)_2$, 0.001 M $MgCl_2$, 0.3 M KCl) for one to three hours. Control eggs were incubated in the same solution but glycerophosphate was omitted. After incubation the eggs were washed in 5 changes of 0.5 *M* KCl and embedded in Araldite 6005. Ultra-thin sections were stained for 30 seconds in phosphotungstic acid and examined in the Philips 100C electron microscope at 60 Kv. The presence of electron-dense lead phosphate precipitates in the sections was taken to indicate acid phosphatase activity.

In the eggs of all three species, acid phosphatase activity was localized in large and small vesicles which are presumably lysosomal in nature. In *Spisula* the fibrous network that lines the vitelline membrane was also acid phosphatase-positive. In *Ciona* acid phosphatase activity was found in what appeared to be a jelly coat lining the surface of the egg and lying in the perivitelline space. In *Echinarachnius* heavy acid phosphatase activity was localized in small vesicles that lined the plasma membrane. Localized acid phosphatase activity was not found in the control eggs. Cortical vesicles of *Spisula* and *Echinarachnius* were not acid phosphatase-positive. Vesicles, similar in ultrastructure to cortical vesicles, could not be found in *Ciona*. The acid phosphatase negativity of cortical vesicles is evidence that they are not lysosomal in nature.

Aided by Training Grant 9 T1 HD 26-03 from the NIH.

Interactions between Limulus polyphemus and two species of marine bacteria. WILLIAM R. SMITH.

The effects of two species of marine bacteria on small *Limulus* (4–8 cm.) were investigated. One, a *Vibrio* species, was isolated from *Limulus* by F. B. Bang; the other, an aeromonad designated 027, was isolated from larval oysters by Haskal Tubiash, and has been shown to be pathogenic for a wide range of marine invertebrates, including clams, fiddler crabs, oysters, and lobsters.

Eleven out of 22 animals died after inoculation with 2.5×10^5 027. Inoculation of 2.5×10^6 027 resulted in death of all the infected animals. Injured animals, maintained in sea water containing $>10^6$ 027/ml. became infected within two hours. Uninjured animals did not become infected over a six-day observation period.

After inoculation with 2.5×10^5 or 2.5×10^6 *Vibrio*, bacteria could be recovered from the hemolymph of infected animals for 24 hours. Inoculation of $>10^8$ *Vibrio* resulted in the death of two out of five animals within 48 hours. Bacteremia persisted in the remaining animals for 96 hours. The hemolymph of animals inoculated with 2.5×10^5 *Vibrio* and maintained at 11°, 17° and 18° C. yielded bacteria for 144 hours, but no deaths occurred within these groups. Injured animals maintained in sea water containing $>10^6$ *Vibrio*/ml. became infected by 12 hours. Uninjured animals did not become infected. *Limulus* sera were found to inhibit the growth of *Vibrio in vitro*.

Vibrio endotoxin, inoculated *in vivo* (0.1 ml.), caused a rapid clearance of hemolymph cells (within five minutes), but did not cause death except when given repeatedly. In contrast, a single inoculation of 0.1 ml. of 027 endotoxin was sufficient to cause death of all the inoculated animals within twenty-four hours.

Fluorescent antimyosin labeling in locally contracted chick myofibrils. R. E. STEPHENS.

Through fluorescent antimyosin labeling of chick myofibrils, Szent-Györgyi and Holtzer have shown an apparent quantitative shift of myosin towards the lateral edges of the A-band on contraction. On the basis of this observation, Szent-Györgyi and Johnson proposed that myosin in one half of a sarcomere is attached contralaterally to the actin of the other half; a shift of myosin towards the lateral edge of the A-band draws in the contralaterally attached actin, resulting in contraction of the sarcomere.

It would be of interest to determine if this apparent shift of myosin in one half of a sarcomere is actually responsible for contraction of the opposite half. If one could observe a sarcomere that has undergone contraction on only one side, antibody labeling on the lateral edge of the A-band opposite the side in which contraction occurred would support the contralateral view. The ultraviolet microbeam technique permits one to accurately irradiate selected areas of sarcomere and locally stop contraction.

Glycerinated stretched chick muscle was homogenized in low-salt buffer and the resulting myofibrils were irradiated with a 1.2 μ beam of 270 mμ ultraviolet light. The preparation was contracted slowly with ATP and then incubated for 20 minutes with fluorescent antimyosin.

Irradiation of a full A-band prevented contraction of the sarcomere and no myosin shift was seen; adjacent contracted sarcomeres clearly showed the effect. I-band irradiation stopped contraction of the irradiated half-sarcomeres but their adjacent halves contracted normally. Irradiation of one-half of the A-band prevented contraction only in the irradiated half-sarcomere. In these partial contractions, the antimyosin labeled the lateral edge of the A-band on the side in which contraction occurred; the opposite half of the A-band showed uniform antibody labeling. These results are in direct disagreement with the Szent-Györgyi-Johnson explanation of the antimyosin labeling anomaly.

Supported in part by grants from the National Science Foundation (GB-2060) and the National Cancer Institute, U.S.P.H.S. (CA 04552).

Band pattern changes in the striated adductor muscle of Pecten irradians. J. W. Sanger and A. G. Szent-Györgyi.

From observations of band patterns of *Pecten irradians* adductor muscle at different lengths, we wish to report that the A-band shortens as the sarcomere length decreases. Muscle length and sarcomere size were obtained at different lengths by opening the shell to various degrees. The muscle was glycerinated *in situ* after the rest of the animal's body was removed. Measurements of sarcomere and A-band lengths with the phase-contrast microscope showed shortening of the A-band from a length of 2.3 μ to 0.8 μ at corresponding sarcomere lengths of 3.2 μ to 1.2 μ. From stretched muscle down to a sarsomere length of 2.3 μ, the I-band decreases and then remains constant on further sarcomere shortening. Observations on myofibrils with low magnification electron microscopy confirmed these results. From the distribution of sarcomere and A-band lengths *versus* length of muscle, it is shown that the apparent A-band shortening is a definite shortening of the A-band and not a reflection of some statistical variation of sarcomere, A-band, and I-Z-band lengths.

Addition of ATP to myofibrils, whose ends are attached to the coverslip, caused a shortening of the A-band. However, when the myofibrils were not attached or were attached at one end only, increased densities were observed at the Z lines on addition of ATP.

Cross-sections of scallop muscle showed the presence of thick and thin filaments in the overlap region and also an H zone. The thick filaments, which have a diameter of 200–250 Å, are surrounded by 10–12 thin filaments.

The sliding filament theory, which has explained band pattern changes in several muscles, is based on the sliding of the thin (actin) filaments past the thick (myosin) filaments composing the A-band; the lengths of the thick and thin filaments remain constant. In scallop, on shortening of the sarcomere, the A-band shortens. Although scallop has two types of filaments, further studies will be needed to show their relationship to the filaments in the muscles studied by Huxley and Hanson.

Ultraviolet microbeam studies of contraction in invertebrate striated muscle. R. E. Stephens.

The Hanson-Huxley sliding filament theory requires constancy of the A-band. Recently it has been shown that the A-band decreases with decreasing sarcomere length in several phyla of invertebrates. Employing ultraviolet microbeam irradiation of individual sarcomeres, one can analyze this anomalous effect.

The adductor of *Pecten irradians* and the extensors of *Homarus americanus* and *Limulus polyphemus* were glycerinated in 50% glycerol-low-salt buffer for 24 hours at 4° C. and then homogenized in low-salt buffer. The resulting myofibrils were contracted after irradiation by perfusion of ATP. In these muscles, full A-band irradiation with 270 mμ UV stopped contraction of the sarcomere. I-band irradiation prevented contraction in the irradiated half-sarcomeres only. Sarcomeres in which two-thirds of the A-band had been irradiated, leaving a lateral edge intact, contracted at the non-irradiated edge. These results, the same as those reported previously by the author for rabbit psoas, are consistent with a sliding mechanism.

In addition, *Pecten*, *Homarus*, and *Limulus* (having 2, 3, and 5 μ A-bands, respectively) contract normally when irradiated at the center of the A-band with a 1 μ microbeam.

Pecten and *Homarus* myofibrils show normal band-pattern changes when under no tension. When *Pecten* was microbeamed longitudinally to prevent contraction in half of the myofibril or when *Homarus* myofibrils adhered to the coverslip, the A-bands shortened on addition of ATP. In free *Limulus* myofibrils, A-bands shorten with ATP but shorten more so under tension. When half of a A-band in an adhering *Homarus* myofibril was irradiated, the irradiated half-sarcomere and its half-A-band remained constant while the intact half-sarcomere contracted and its half-A-band shortened.

It can be concluded that these muscles operate by a sliding mechanism when under no load but under tension a unique form of contraction takes place which involves A-band shortening.

Supported in part by grants from the National Science Foundation (GB-2060) and the National Cancer Institute, U.S.P.H.S. (CA 04552).

Studies of the ribosomal proteins of Arbacia punctulata eggs. DAVID SULLIVAN.

Some observations on the ribosomal proteins of unfertilized *Arbacia* eggs are reported here as a preliminary to a study of the changes, if any, in the composition of the ribosomes in the course of development. As yet, very little is known about ribosomal proteins. Ribosomal pellets, from post-mitochondrial supernatants of homogenates (in a solution of 0.05 M Tris pH 7.5, 0.02 M KCl, 0.004 M $MgCl_2$ and 0.005 M mercaptoethanol) were cleaned either by centrifugation through a sucrose density gradient or by resuspending in the homogenizing medium and recentrifuging at 105,000 g for one hour. In the analytical ultracentrifuge this preparation showed peaks of 75S, 50S and 25S. Attempts at solubilizing proteins from the above preparation were made by extraction at low pH, using formate buffers ranging in pH from 2.0 to 6.0 and in concentration from 0.01 M to 1.0 M after activation of latent ribosomal RNase as described by Spitnik-Elson (1964) for *E. coli*. These attempts failed to bring any detectable protein into solution, at least as determined by starch gel electrophoresis followed by staining with amido black. On the other hand, in 0.1 M barbital buffer at pH 8.8, 25% of the ribosomal protein was soluble. Starch gel electrophoresis on this fraction (at 100 V, 25 ma for 20 hours in gels 18 cm. × 11 mm. × 6 mm.) showed one major component and a slightly more slowly moving band. Both bands migrated toward the cathode. Analytical ultracentrifugation of the soluble fraction showed two peaks that had sedimentation coefficients of 10 and 25. The relative homogeneity shown by this extracted protein fraction is in sharp contrast to the heterogeneity of the ribosomal proteins of *E. coli*.

Aided by Training Grant 9 T1 HD 26-03 from the National Institutes of Health.

The uptake of ethylenediamine tetraacetic acid by pure cultures of marine plankton algae. W. ROLAND TAYLOR.

Chelating reagents such as EDTA are commonly added to both synthetic and sea water media for marine algae. Experiments using carbon-14-labeled EDTA were undertaken to determine if the organic component of the chelate complex enters the cell. Bacteria-free cultures were grown in the enriched sea water medium of Guillard to which had been added 0.15 microcurie of labeled EDTA. The organisms used were: *Synecoccus* sp., *Dunaliella tertiolecta*, *Amphidinium carterae*, *Cyclotella nana*, *Monochrysis lutheri*, and *Isochrysis galbana*. All organisms took up the isotope. The isotope was not removed by exhaustive washing with unlabeled EDTA, indicating chemical combination rather than physical adsorption. In terms of isotope per cell, the chrysomonads and the dinoflagellate contained the greatest amount of activity, the diatom was intermediate and the blue green and green algae were lowest. Ultrasonic rupturing of washed cells followed by high speed centrifugation showed appreciable amounts of carbon-14 in the soluble fraction. The amount varied with the species, the chrysomonads having about 75% of the activity in this fraction, while in *Amphidinium* and *Cyclotella* it amounted to only 20%. Other species were intermediate. The soluble fractions from *Dunaliella* and *Synecoccus* were further fractionated using hot trichloroacetic acid. Half of the total soluble carbon-14 activity was found in the protein precipitate.

It is concluded that the organic portion of the chelate metal complex is taken up by these six algal species. Experiments are in progress to determine the chemical nature of the carbon-14 activity found in the various cell fractions.

Partially supported by ONR and AEC contracts with the Johns Hopkins University.

Chromatographic analyses of plant pigments in intertidal sediments. W. Roland Taylor and Conrad D. Gebelein.

Four areas of different sediment type were chosen on Barnstable harbor flats. Duplicate cores were taken on four occasions. Cores were sectioned immediately into the surface millimeter and five serial centimeter sections. The sections were extracted twice with methanol and chromatographed according to Jeffreys (1960). The per cent organic content was determined by ashing dried sediment at 420° C. to constant weight. Sediments of Area 1 are well sorted sands. This is Station D of Sanders (1962). Sediments of Area 2 are flocculent mud. Sediments of Area 3 have a high silt/clay ratio and are characterized by *Amphitrite* sp. Area 4 is characterized by high concentrations of migrating benthic diatoms. The highest concentration of total pigment is always found in the upper 1.1 cm. Large quantities of pigment are found to 5 cm. Concentrations of chlorophyll *a* in the surface millimeter and first centimeter, respectively, are: Area 1, 227 and 515 mg./m.^{2}; Area 2, 450 and 331 mg./m.^{2}; Area 3, 530 and 290 mg./m.^{2}; Area 4, 285 and 442 mg./m.^{2}. Chlorophyll *a* concentrations decrease 60–70% from one to five centimeters; fucoxanthin decreases 40–50%; carotenes, diatoxanthin and diadinoxanthin remain constant or increase slightly. Ratios of pigments from different locales vary similarly: chlorophyll *a*/chlorophyll *c* and chlorophyll *a*/fucoxanthin are very constant with depth; chlorophyll *a*/carotenes and chlorophyll *a*/xanthophylls decrease markedly. No correlation was found between phaeophytin and chlorophyll *a*, as phaeophytin was found only in the lower 2 cm. of the diatom and *Amphitrite* fields. However, the amount of completely degraded, methanol-extractable material increases with depth in all cases. No correlation appears between the per cent organic content of the sediments and the concentrations of chlorophyll *a*.

Partially supported by AEC and ONR contracts, and NIH training grant 5T1 GM 535-04.

The effects of actinomycin D on head regeneration in a brackish-water ciliate, Tracheloraphis sp. Reuben Torch.

Previous work has established that fragments of *Tracheloraphis* sp. are capable of extensive regeneration, even in the absence of the nuclear apparatus or any of its parts. This indicates that the cytoplasm contains at least some preformed information, and in view of the lack of cytochemically detectable macronuclear DNA, the possibility exists of information encoding different from current theory.

Organisms were placed in shallow culture dishes containing 0.5 ml. of actinomycin D in concentrations of 0, 10, 24, 45, and 82 μg./ml. of filtered (Millipore AP20) brackish water and immediately decapitated by means of glass needles. Following complete regeneration of the heads (6–8 hours), the animals were decapitated again; and after regeneration, they were decapitated again, and so on. At the highest concentration of actinomycin D, animals are able to regenerate heads for approximately 48 hours (6 regenerations), after which they lose their regenerative capacity. After several decapitations in high concentrations of actinomycin, it is not unusual to observe anomalies, such as lateral rather than anterior head regeneration. At lower concentrations of the drug, the animals appear to be unaffected and can regenerate after additional decapitations. Animals placed in 82 μg./ml. of actinomycin for 48 hours *prior* to decapitation are incapable of regeneration.

The data suggest that in the presence of high concentrations of actinomycin D, the organisms ultimately exhaust the information necessary for head regeneration. This is consistent with the concept of a stable messenger RNA produced by nuclear DNA (if it exists in this organism), but does not exclude the possibility of information encoding by nuclear and/or cytoplasmic RNA. The effects of actinomycin D on nucleic acid and protein syntheses in normal and regenerating organisms are being studied.

Supported by a grant from the NIH (GM 11252 02).

DNA in Arbacia. Walter Troll, Louis Fishman, Stephen Jaffe and Joel Chasis.

The mature haploid egg of the sea urchin is unusual in its DNA content and distribution. It contains between 50 and 100 times the quantity of DNA observed in the sperm cell and all but 1–2% occurs outside the nucleus. The function of this DNA is not known and we felt the isolation of egg DNA and comparison with sperm and somatic DNA would be of interest.

We developed a general method for isolating DNA, using Sephadex G-200 gel filtration, after removing most of the proteins by shaking with chloroform and isoamyl alcohol and reducing the size of RNA present by treatment with RNAase. DNA is separated into the outside volume of a Sephadex G-200 column, while most other materials, including RNAase, are eluted in the inside volume.

The material obtained from eggs and plutei showed marked hyperchromicity on heating, but did contain an impurity of tightly bound protein. Comparison of the melting properties of sperm, eggs, and plutei (somatic DNA) revealed virtual identity of egg and sperm DNA with Tms of 68 and 68.5, respectively, while the plutei DNA showed a Tm of 70.5. This small difference might be a consequence of the protein impurity occurring in the plutei DNA.

The isolation of DNA from haploid eggs provides the opportunity for further study of the function of this non-genetic DNA.

Incorporation of H^3-thymidine and H^3-uridine by oocytes of Pectinaria gouldii. Kenyon S. Tweedell.

Developing oocytes were exposed *in vivo* by intracoelomic injection of 5 to 10 μC. of H^3-thymidine (1.9 C./millimole) or H^3-uridine (1.7 C./millimole) per animal. Pulse times ranged from 15 minutes to 72 hours, with egg harvests to 7 days. Alternatively, the oocytes were shed directly into filtered sea water containing 10 μC./ml. of the labeled precursors.

After injection the oocytes were shed, fixed and flattened on coverslips under pressure, or the entire animals were fixed, embedded and sectioned at 5 μ. Oocytes shed directly into the isotopes were mounted on coverslips. Egg harvests were made at ½, 1, 2, 4, 8, 16 and 24 hours and on the 2nd, 3rd and 7th day after exposure. Both compressed whole oocytes and sections were prepared for autoradiography by dipping in Kodak NTB2 or Ilford G-5 emulsion.

Injection of H^3-thymidine for one to 72 hours produced heavy labeling in scattered dividing cells within the ovary. After one hour, the smallest coelomic oocyte packets showed a heavy label primarily in the cytoplasm. At the end of 2, 3 and 7 days, a few of the larger cell packets, presumably resulting from cell growth, showed heavy cytoplasmic label and little nuclear uptake. During development the cell packets fragment and the individual oocytes enlarge. Neither growing nor mature primary oocytes showed any label *in vivo,* even after 7 days. Mature oocytes shed directly into H^3-thymidine, either before or after germinal vesicle breakdown, failed to show any incorporation. Again, only the smallest packets of oocytes became labeled.

All cell stages incorporated H^3-uridine even at the shortest pulse. The intensity of the label was proportional to the pulse length. Diffuse labeling occurred over all ovarian cells. All oocytes still in packets appeared to be labeled only on the cytoplasm. Developing single oocytes as well as mature oocytes were lightly labeled over the cytoplasm but possessed a heavy nuclear label, including a heavy ring around the nucleolus. After a prolonged label (6-day) some of the mature oocytes became heavily labeled in the cytoplasm as well.

The effects of various bacterial and viral inhibitors on the development and metabolism of fertilized sea urchin eggs. Dina Van Praag, Eric J. Simon and Herbert S. Rosenkranz.

This study was undertaken to investigate the effects of known anti-bacterial and anti-viral agents on the metabolism of differentiating cells.

The incorporation of C^{14}-uracil, H^3-thymidine and C^{14}-valine into the acid-insoluble fractions of the developing *Arbacia* embryo was used as an index of RNA, DNA and protein syntheses, respectively. By these criteria, it was found that phenethyl alcohol (PEA), an inhibitor of bacterial messenger RNA, halted the synthesis of both DNA and RNA by 50% when a PEA concentration of 0.046% was used. A similar inhibition of protein synthesis required a PEA concentration of 1.78%. However, exposure of the fertilized eggs to low levels of PEA (~0.0013%) led to the development of abnormal embryos. These data are consistent with an action of PEA on the messenger RNA of these embryos.

The bacteriostatic agent hydroxylamine was also a more effective inhibitor of DNA than of RNA and protein syntheses. Concentrations of 2.2×10^{-5}, 8.9×10^{-5} and 1.4×10^{-3} *M*, respectively, were required to inhibit these macromolecular syntheses by 50%.

The anti-viral agent, isatin thiosemicarbazone, reduced DNA synthesis by 50% when a concentration of 0.55 μg./ml. was used. RNA synthesis was blocked to the same extent with 1.85 μg./ml. while protein synthesis proceeded even in the presence of 40 μg./ml.

Dinitrophenol and levallorphan, two inhibitors of bacterial ribosomal RNA, were effective in suppressing protein and DNA syntheses, respectively.

This study was supported by the U.S.P.H.S. (AI 05111 and MH 04294), the American Cancer Society and the Office of Naval Research (Nonr 266-89).

Seasonal and diurnal fluctuations in the quantity of dissolved carbohydrate in Oyster Pond, Cape Cod. GERALD E. WALSH.

Oyster Pond is a large (volume = 750,000 m.^{3}) oligohaline coastal pond which communicates with Vineyard Sound through a smaller pond and is slightly affected by tidal action only at spring tide. Studies were conducted from 2 January to 27 July 1964, to learn if a seasonal cycle in dissolved carbohydrate exists, and, if so, to correlate this with physical and chemical parameters of the pond. Water samples were taken at surface, mid-depth, and bottom in a basin six meters in depth, between 0800 and 0900 hours, at approximately one-week intervals, and analyzed for dissolved carbohydrate (DCHO), chlorophyll *a*, chlorinity, dissolved oxygen, pH, Eh, and temperature. Similar analyses, with the exception of Eh and chlorophyll *a*, were made on surface water at three-hour intervals from 0600 on 31 July to 0600 on 1 August. Rate of carbon assimilation and DCHO production was obtained by light- and dark-bottle experiments during the 24-hour study.

Seasonal analyses indicate that Oyster Pond is eutrophic, exhibiting a clinograde oxygen curve and development of a tropholytic zone four weeks after overturn. Values for pH and Eh were similar to those of eutrophic lakes. DCHO concentrations in surface and mid-depth waters varied between 1.48 and 2.71 mg./L. Lowest values occurred between 12 February and 16 March, when chlorophyll *a* concentration rose from 2.68 to 21.71 μgm./L. At the bottom, during summer stagnation, DCHO concentration increased from an average of 1.53 to 3.97 mg./L.

In the diurnal study, DCHO concentration increased from 1.67 to 3.33 mg./L. between 0600 and 1800, and subsequently dropped to 1.59 mg./L. at 0600 the next day. Rate of carbon assimilation peaked between 1500 and 1800 when an average of 371.3 mg./m.^{3}/hr. was assimilated. DCHO production was greatest between 1200 and 1500. At that time, 450.0 mg./m.^{3}/hr. were released. Utilization of DCHO at night explains why low concentrations of DCHO were found the following morning during a phytoplankton bloom.

It is concluded that DCHO concentration in Oyster Pond was related to decomposition of organic matter in sediment and to intensity of photosynthetic and other metabolic activity of primary producer organisms.

Supported by Grant GB-561 from the National Science Foundation.

Glucose stimulation of insulin release from toadfish islet tissue in vitro. DUDLEY WATKINS, J. LEONARDS, P. K. DIXIT, S. J. COOPERSTEIN AND ARNOLD LAZAROW.

We have examined the effect of glucose on the release of insulin from toadfish islet tissue *in vitro*. In contrast to mammalian pancreas, toadfish islet tissue is segregated into one or more discrete bodies which are relatively free of acinar tissue. Islets were decapsulated and preincubated for 10 minutes in either 0.140 *M* NaCl (containing 10^{-3} *M* Na_2HPO_4-KH_2PO_4 buffer, pH 7.0) or Krebs-Ringer phosphate (pH 7.0). They were then rinsed briefly in fresh media, transferred to microtubes containing 100 μl. of test medium, and incubated for one hour. After incubation the islets were removed and the incubation medium was assayed using the rat epididymal fat pad method.

When islets were incubated in the saline medium, less than 6 milliunits (mU) of insulin were released per mg. of islet. Whereas the addition of glucose (300 mg.%) had no significant effect at 0° C., at 25° C. glucose produced a three-fold increase in the amount of insulin released (average of 17 experiments was 21.6 mU/mg. islet, $p < 0.001$). When the Krebs medium was used at 25° C. the value was 7.7 mU/mg. islet (5 experiments); addition of glucose increased this to 16.2 mU/mg. islet (6 experiments). The presence of gelatin (100 mg.%) in the medium inhibited the glucose effect.

These results demonstrate that under proper conditions glucose stimulates the release of insulin from toadfish islet tissue. This system, using the isolated toadfish islet, should prove useful in studying the mechanism of insulin release.

Supported by Grants AM 6049, AM 00824, and AM 09059 from the National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S.

The effect of localized ultrasound on the isolated sartorius muscle fiber of frog.

WALTER L. WILSON, FLOYD J. WIERCINSKI AND RONALD M. SCHNITZLER.

Ultrasound applied locally to the surface of a nerve of a frog sciatic-gastrocnemius preparation evokes a response in the nerve as evidenced by contraction of the muscle. An isolated whole muscle fiber in unbuffered Ringer solution fails to respond with a twitch to ultrasound applied directly to its surface. Ultrasound does, however, produce movement and an injury reaction in the muscle fiber.

Ultrasound was applied to the fiber surface by means of a needle machined into the tip of a stainless steel acoustic horn. The horn was cemented at its base to one end of an electroded and polarized barium titanate hollow cylinder. This transducer was activated at approximately 85,000 cycles per second. The maximum peak-to-peak amplitude of the needle tip was 10 μ.

When ultrasound is applied to a fiber in Ringer solution, there is a breakdown of cross-striations in the region of the needle tip and a slow movement of sarcoplasm toward the region of the injury. At cessation of treatment this slow movement ceases and reverses direction. The injury reaction proceeds across the fiber and along its length.

When a fiber is immersed in paraffin oil, calcium-free Ringer, or in an isotonic solution of KCl, NaCl, or Na oxalate, treatment of the fiber with ultrasound does not evoke an injury reaction or movement of the sarcoplasm, although some myofibrils become prominent. If any one of these solutions is replaced by unbuffered Ringer, or if calcium ion is added to any one of these solutions, movement and injury can now be evoked. These effects occur also in $CaCl_2$ solutions as dilute as 0.001 *M*. This response to ultrasound appears to be calcium ion-dependent and apparently ultrasound increases membrane permeability to calcium ion.

Supported by Grant GM 08775-03, N.I.H.

Vol. 127, No. 3 December, 1964

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

TUBE-BUILDING AND FEEDING IN THE CHAETOPTERID POLYCHAETE, SPIOCHAETOPTERUS OCULATUS

ROBERT D. BARNES

Department of Biology, Gettysburg College, Gettysburg, Pennsylvania

Chaetopterus variopedatus is one of the best known polychaete annelids. The construction of the familiar U-shaped tube of this worm was first studied by Enders in 1908. Enders in a later paper (1909) made some observations on feeding in *Chaetopterus* but failed to recognize the use of the mucous bag. Complete understanding of the feeding mechanism in *Chaetopterus* was provided by the studies of MacGinitie in 1939. Much less is known about the behavior and physiology of other chaetopterid genera. Only a few casual observations of the North Pacific *Mesochaetopterus rickettsii* and *Phyllochaetopterus prolifera* have been reported (MacGinitie and MacGinitie, 1949).

The purpose of this study was to investigate the mechanism of tube-building and feeding in *Spiochaetopterus,* a chaetopterid genus which is rather more typical of the family than is *Chaetopterus.* The species studied was *Spiochaetopterus oculatus* Webster, a very common member of the genus along the North Atlantic coast.

This investigation was carried out at the Duke University Marine Laboratory at Beaufort, North Carolina, during the summer of 1963, and was supported by a National Science Foundation Summer Study Grant. The author wishes to express his appreciation to the Duke University Marine Laboratory for the facilities extended to him during the course of this study. Acknowledgment is also due to Dr. Marion Pettibone of the United States National Museum for her kind assistance and suggestions.

MATERIALS AND METHODS

Worms were collected within the tube, care being taken to dig out the entire tube from the substratum. In studying tube construction, specimens were restricted to a short section of the natural tube, the other part having been cut away. The section containing the worm was then placed in the upper part of a piece of glass tubing having a bore of 1.5 mm. Worms were also entirely removed from their own tube and placed within glass capillary tubes. Transfer was brought about by placing the tube of the worm within the end of a capillary tube (0.8–

Copyright © 1964, by the Marine Biological Laboratory

1.2 mm. in diameter) which just exceeded the diameter of the worm tube. A dissecting needle was then rolled down the length of the worm's tube, driving the worm out and into the capillary tube. Since removal of the worm caused some damage to the end of the body just in front of the dissecting needle, care was taken to always drive the worm forward. Thus, any damaged segments would be at the posterior end of the body.

The glass tubes containing worms were kept in a vertical position by placing them in a glass cylinder, where they were held against the inner side of the cylinder wall with a large plug of glass wool wrapped in a piece of black cloth. The black cloth provided a good background for visual observations. A short piece of glass tubing having a diameter of 2 cm. was extended through the center of the plug of glass wool to provide for circulation of water in the lower part of the cylinder. The entire cylinder, except during periods of observations, was then submerged in a battery jar of running sea water.

The worms were observed in a vertical position through the glass wall of the cylinder. Observations were made with a binocular dissecting microscope (from which the base had been removed) suspended horizontally by the arm from a clamp attached to a ring stand.

RESULTS AND OBSERVATIONS

Spiochaetopterus oculatus is a small tube-inhabiting chaetopterid polychaete, varying in length from 3.0 to 6.0 cm. Like many other members of the family Chaetopteridae, the body is divided into three regions. The broad anterior region (Fig. 1, A) is composed of a poorly defined head followed by nine segments. The parapodia of these segments are limited to a short notopodium, each of which is provided with a fan of capillary setae. The fourth notopodium is peculiar in possessing a giant blade-like seta in addition to the capillary bundle.

The cylindrical middle region of the body (Fig. 1, C) is composed of 18 to 37 segments, although 21 to 24 segments are most commonly present. Each of these segments is provided with distinctive parapodia composed of a foliaceous notopodium and an uncinate neuropodium. The foliaceous notopodium will be described in detail later.

The terminal body region is made up of a large but varying number of segments. It is somewhat similar to the middle region of the body except that the notopodia, rather than being foliaceous, are reduced to antenna-like processes (Fig. 1, D).

The tube

The tube of *Spiochaetopterus* is a vertical annulated structure composed entirely of a secreted cornified, or chitin-like, material. The tube is built vertically in the substratum with from 2 to 10 mm. of its length projecting above the surface. The total length of the tube varies greatly, and this variation is perhaps related to the nature of the substratum. At Beaufort, North Carolina, *Spiochaetopterus* is most abundant in a muddy substratum, where it occurs from below the low-tide mark through the lower third of the intertidal zone. In such habitats the worm may have a density as great as 25 to 35 individuals per square foot. In sandy

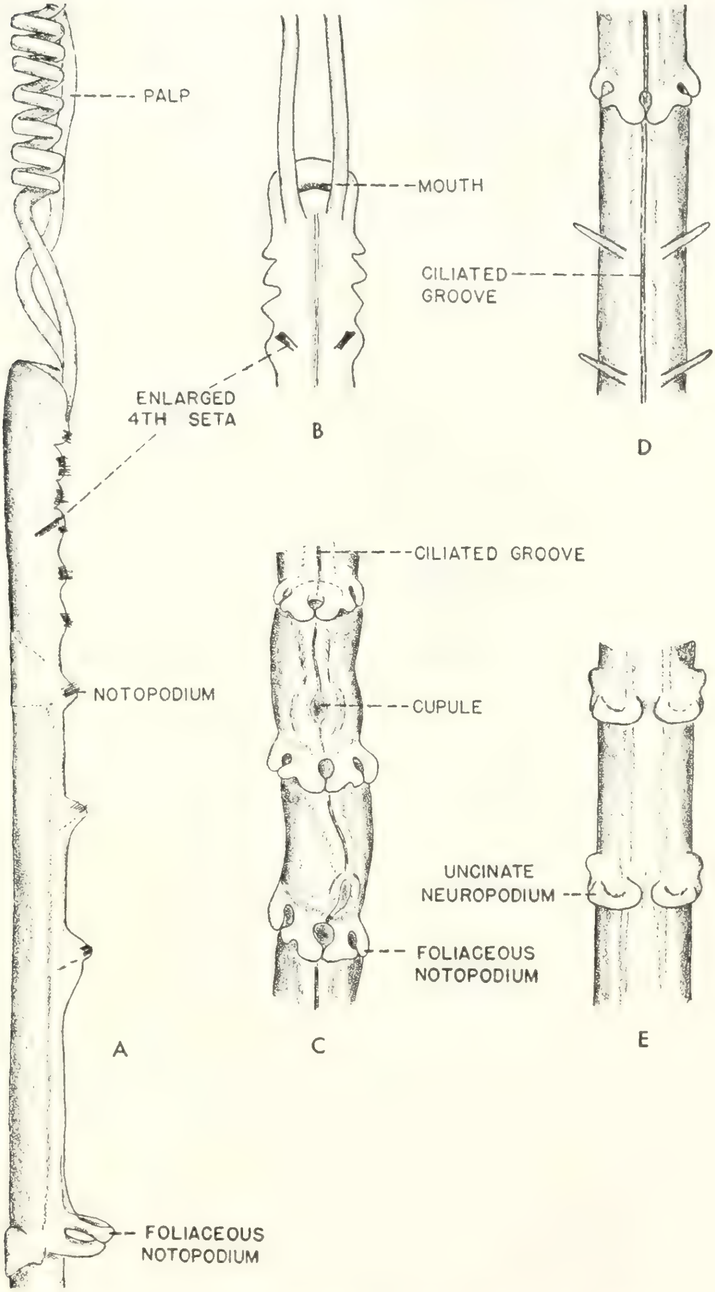

PALP
MOUTH
CILIATED GROOVE
ENLARGED 4TH SETA
B
D
CILIATED GROOVE
CUPULE
NOTOPODIUM
UNCINATE NEUROPODIUM
FOLIACEOUS NOTOPODIUM
A
C
E
FOLIACEOUS NOTOPODIUM

FIGURE 1. External structure of *Spiochaetopterus oculatus*. A. Lateral view of anterior body region and first foliaceous notopodium of middle body region. B. Dorsal view of anterior end of body. C. Dorsal view of three segments of middle body region. D. Dorsal view of last segment of middle body region and first two segments of posterior body region. E. Ventral view of two segments of middle body region showing uncinate neuropodia.

substrata the species is much less abundant and careful searching may be necessary to locate the tubes.

In several muddy habitats in the Newport River Estuary, from which specimens were regularly collected, the total length of the tube mostly ranged from 8.0 to 12.0 cm. However, on sand flats tubes were obtained which reached a length of more than 50 cm.

The diameter of the tube is not nearly so variable as its length. The internal diameter is generally about 0.7 mm., although one had an internal diameter of 1.2 mm. The wall is thickest, about 0.03 mm., in the upper extent of the tube. The tube in this region (Fig. 2, A) is tough, rigid, markedly annulated, and opaque to blackish in color. The annulations are formed by a slightly thicker area of tube wall and are spaced 1.0 to 1.5 mm. apart.

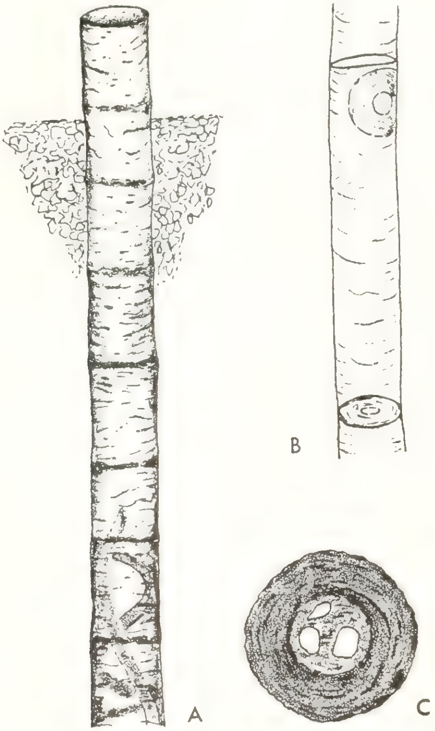


FIGURE 2. Tube structure. A. Upper end of tube showing relationship of opening to substratum. B. A short section of the lower region of the tube showing an old partition (top) plastered against the wall of the tube and a functional partition (bottom) in place. C. Surface view of a partition. Note thinner central area perforated by three openings.

Since the oldest part of the tube is generally the upper portion, the tube wall tends to become thinner as it extends downward into the substratum. The lower part of the tube is colorless and transparent, with no noticeable annulations and with thin, easily collapsible walls.

At some point near the bottom of the tube, and always within the thin transparent section, the lumen is obstructed by a partition (Fig. 2, B). The partition is shaped somewhat like a button (Fig. 2, C). The peripheral portion is relatively thick and joins with the tube wall. The circular central area is thinner and perforated by one to four openings. The bore of the tube is thus never completely blocked, and the openings in the partition permit continual water circulation. The partitions are periodically removed and new ones constructed at different levels. The old partitions when removed are plastered against the inner side of the tube wall, and can be observed all along the length of the more transparent lower quarter of the tube. The function of the partitions is not certain. They may serve to prevent the thin walls of the lower part of the tube from collapsing.

Mechanism and movement within the tube

Like all chaetopterids, the activities of *Spiochaetopterus* are limited to the confines of its tube. Yet worms observed within glass tubes or within the transparent sections of their own tubes exhibited considerable facility of movement. The body of the worm maintains contact with both sides of the tube wall. On one side of the tube this contact is supplied by the convex ventral surface of the anterior region of the body and by the uncinate neuropodia (Fig. 1, E) of the middle and posterior regions of the body. These neuropodia have the form of a pair of slightly curved shelves.

The opposite wall of the tube is contacted by the notopodia of the anterior and middle body regions. The anterior notopodia are slightly tapered processes having a bundle of capillary setae. The notopodia of the middle section are foliaceous and each consists of two parts as shown in Figure 1, C. The foliaceous processes are normally held at right angles to the body so that their distal margins touch the wall of the tube.

The worm may be oriented within the tube with the head directed upward or downward, but most commonly it assumes an upward position. In either position the worm can readily move up or down the length of the tube. Slow upward movement appears to be largely brought about by the leg-like action of the anterior notopodia. These appendages push against the side of the tube, one notopodium acting alternately with the notopodium on the opposite side. The setae bundles are extended and pushed out and downward against the tube wall. Most conspicuous are the 7th, 8th, and 9th notopodia which are the largest notopodia in the anterior part of the body. The action of these notopodia involves not only extension but an outward turning of the anterior margin.

In slow downward movement, the worm appears to release contact with the tube wall and slowly sink.

Rapid movement involves body contraction and is essentially peristaltic in nature. The uncinate neuropodia of the middle and posterior body regions appear to provide the principal means of anchorage in this type of movement.

A worm can easily reverse itself within the tube. In changing from a head-up to a head-down position, the anterior end is bent downward and then moves down the tube against the posterior part of the body, which is simultaneously moving upward in the opposite direction. The turning point of the body thus remains more or less at the same level. Reversing from a head-downward position may be carried out as just described, with the anterior end moving upward through the tube, or the worm may perform a reverse jackknife, in which the posterior end turns downward and moves down the tube.

Tube construction

Secretions for tube construction are produced by epidermal glands which open onto the ventral surface of the anterior region of the body. This secretory surface is large. It extends from near the anterior margin of the head through the entire anterior body region, *i.e.*, to the level of the 9th parapodia, and covers the entire convex ventral side of the body. It is hoped that the histology of the glandular area, as well as other aspects of the histology of *Spiochaetopterus*, will be described in a later paper.

Observations on tube construction were made on worms confined to a very short section of the original tube, the remainder of the tube having been removed. An animal so confined immediately began the construction of additions to the original tube. Such additions were made to either end of the tube but most commonly were initiated at the upper end.

The actual laying down of tube wall is preceded by "exploratory" movements of the anterior end of the body. The anterior end is extended out of the tube as far as the 7th parapodia or a lesser distance and then withdrawn. This movement may be repeated many times with the body often rotating to cover a new arc at each extension. The palps during this time are extended downward between the foliaceous notopodia.

At the time of secretion the anterior end is slowly extended out of the tube, rotating slightly side to side in the process. The projecting anterior end appears markedly turgid with the front margin flared. The body extends out of the tube as far as the 7th parapodia and when this level is reached, the head bends dorsally at right angles to the body. The flexure is located at about the level of the 4th parapodia. At the end of this movement (Fig. 3, A) which may take 10 to 15 seconds, the body is quickly withdrawn, leaving behind and in place a delicate one-half cylinder attached to the older section of the tube. The length of the new addition is equivalent to the distance between the flexure and the level of the body at the mouth of the tube.

The worm now rotates 180° and repeats the secretory projecting movement to lay down another half-cylinder of tube. The second half-cylinder is attached to the first half to form a complete cylindrical addition or section of tube. Such a section would approximate an annulated section in an older part of the tube.

With the completion of the cylinder, the worm now moves up and down applying the ventral secretory surface of the body against the inner surface of the new addition, reinforcing the tube wall with additional secretions. These reinforcing movements may be coupled with exploratory movements, preceding the secretion of another section of tube. Within the period of an hour several new sections may be added to the tube.

Having added to the top end of the tube, the worm may reverse itself and begin additions to the lower end of the tube. Under natural conditions, additions are probably made chiefly to the lower end of the tube.

The reinforcing of the inner wall of the tube with additional secretions is apparently a continual process and would account for the gradual thickening of the tube wall between new and older sections. The convex ventral surface is always pressed against the tube surface.

The transverse partitions which partially block the lower part of the tube are secreted by the head region. The worm reverses itself in the tube if previously directed upward, and then moves downward toward the lower part of the tube. At the level at which the partition is to be placed, the worm halts its downward movement. The head is flexed slightly inward (dorsally) and then rapidly rotated. It is during this rotation, which lasts only about five seconds, that the partition is secreted. The partition is at first shaped like a washer, having a large hole in the center. The hole results from the absence of glands along the front median margin of the head. This area of the head is located in the center of the partition during the rotating movement and at the end of the rotation protrudes through the partition opening (Fig. 3, B).

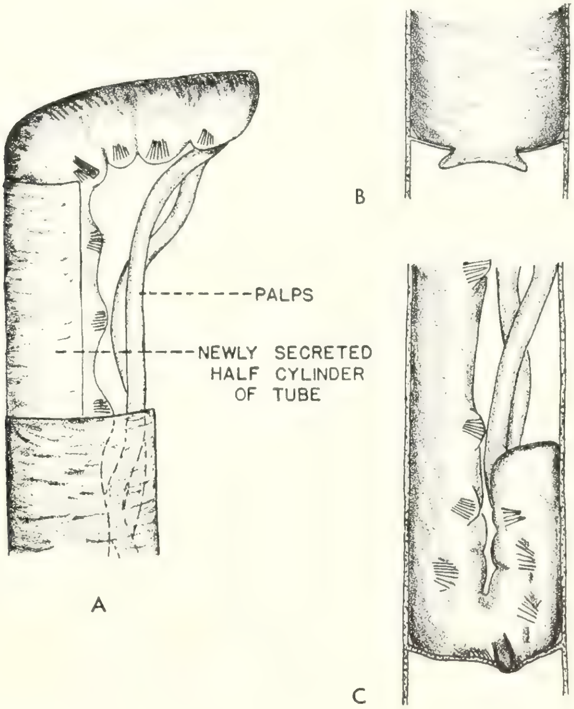


FIGURE 3. Tube construction. A. Worm secreting addition to tube. B. Head of worm secreting first part of a partition. Anterior median region of head is forced through the initially open central area of partition. C. Worm in process of removing a partition by cutting its junction with the tube wall with the heavy blade-like 4th seta.

Following the initial secretion of the partition, the head is flexed and the worm moves the glandular surface back and forth over the partition. This final action apparently reinforces the partition and probably accounts for the reduction of the original opening to two to four small perforations. It is difficult to understand why the original opening would not be completely sealed over.

As additions are made to the lower part of the tube, the tube partitions are continually removed and replaced at lower levels. In removing a partition the worm moves head downward to the site of the partition. The body is flexed at the level of the fourth parapodia and then twisted slightly at the level of flexure so that the right side of the body is closer to the periphery of the partition than is the left. The large, heavy, blade-like 4th seta is now extended downward and then retracted. With each downward stroke, the truncate blade of the seta cuts through the partition where it joins the tube wall (Fig. 3, C). Between each cut the body rotates slightly counterclockwise, and the seta performs another cutting stroke. When the worm makes a full circle, the partition is completely severed from the tube wall. The partition is now pushed down and back against the inner surface of the tube wall, where it is gradually plastered to the wall as additional secretions are laid down. Such old partitions can be observed along the length of the lower part of the tube, but as the tube wall becomes thicker and darker, the incorporated partitions become invisible.

The left 4th seta was never observed being used for cutting a partition. However, in view of the fact that the left seta is as highly developed as the right, it is likely that either seta can be employed in cutting partitions.

In addition to removing tube partitions, the enlarged blade-like 4th setae are also used to slit open the side of the tube to permit the formation of an entire new extent of tube. In initiating such a new construction, the worm makes an irregular transverse cut through the tube wall. The anterior body region is pushed through the rupture and begins the laying down of new tube as already described. The old lower section of the tube is no longer used, and the entrance to it is gradually sealed off.

Such a major alteration was observed only at the lower part of the tube. Whether an entire new tube could be rebuilt in this manner is not known. However, when specimens with obvious new side branches to the tube were found in the field, the new branch was always located in the lower part of the tube.

Observations on tube construction were not limited to the building of additions to an existing section of the tube. Worms observed in capillary tubes would secrete normal additions to either end of the glass tubing, particularly the lower end. Partitions were also constructed and removed within the capillary tubes.

In contrast to *Chaetopterus*, *Spiochaetopterus* is capable of burrowing and rebuilding its tube when removed from the original one. Specimens were removed from their tubes and placed on the surface of a mud-sand substratum within a small beaker. The worms immediately began to burrow downward head first and very shortly disappeared below the surface. Within an hour palps were visible projecting from the mouth of an opening at the surface of the substratum. When excavated, a delicate short tube was uncovered.

Feeding

Most of the observations on the feeding mechanism of *Spiochaetopterus* were made on worms placed in capillary tubes and fed a suspension of carmine particles in sea water or a suspension of stained Pablum.

Spiochaetopterus, like many sedentary polychaetes, is a ciliary feeder. It has been suggested that the grooved ciliated palps might be the principal structures used for obtaining food. This was not found to be true. The palps are primarily used for removing waste and other unwanted materials from the tube and only secondarily as organs of ingestion.

The feeding mechanism is basically like that of *Chaetopterus* (MacGinitie, 1939), in that a water current is passed through a mucous bag, straining out suspended particles, but the process differs from that in *Chaetopterus* in a number of important respects. As described earlier, the middle region of the body of *Spiochaetopterus* bears very distinctive parapodia (Fig. 1, C), each composed of a foliaceous notopodium and an uncinate neuropodium. The foliaceous notopodium is composed of a dorsal and a ventral division. The larger dorsal division bears two lobes, one of which is curved toward the mid-dorsal line; the other is curved ventrally. When held erect, *i.e.,* at right angles to the body axis, the divisions and lobes of the two notopodia so appose each other that they form three ring-like openings, one mid-dorsal and two dorso-lateral (Fig. 4). The mid-dorsal ring is formed by the two incurved lobes from each dorsal division. Each lateral ring is formed by the ventral division and the ventrally curved lobe of the dorsal division.

The inner margins of the rings are lined by large membranelles, which are visible even at a magnification of 30 ×. The effective stroke of each membranelle is directed posteriorly but does not occur in all membranelles of a ring simultaneously. Rather, the beat occurs as a sequence of several waves, which progress around the ring in a clockwise direction.

The beating of the membranelles within the notopodial rings drives water through the tube. The notopodia of the middle body region are held outstretched at right angles to the body axis, and their outer margins touch the tube wall. The ventral body surface with its short uncinate neuropodia is in contact with the opposite wall of the tube. Thus, all water must pass through the ciliary notopodial rings. At the level of any one parapodium there would be three currents, one dorsal and two dorso-lateral, corresponding to the three notopodial rings (Fig. 4).

A mid-dorsal ciliated groove traverses all three regions of the body and terminates just behind the mouth. The groove in the middle region of the body thus crosses the inner wall of the mid-dorsal ring. Behind each ring the groove widens to form a ciliated cupule, except for the first segment of this body region, which lacks a cupule. Anteriorly the cupules are large, well-developed, and located some distance back from the preceding ring. Posteriorly they become progressively less conspicuous and located closer behind the preceding mid-dorsal notopodial ring.

The water current passing through the tube contains suspended detritus and plankton upon which the worm feeds. Although the water current, which thus functions as a feeding current, is created by all of the notopodial rings, the mid-dorsal ring is more directly involved in feeding. Each of these mid-dorsal rings (at least anteriorly) secretes a mucous bag, the end of which is caught and rolled up as a food ball by the cupule located behind the ring (Fig. 4). New mucous film is continuously being produced by the notopodial ring during the feeding process. All of the water passing through a mid-dorsal ciliary ring must also pass through the mucous bag secreted by that ring. The food particles strained out and collected in passage are consolidated as a food ball as the bag is rolled up by the cupule.

Active feeding, *i.e.,* the secretion of mucous bags, is more or less simultaneous and appears to be limited to the anterior segments of the middle body region. The greatest number of mucous bags and food balls ever observed at one time was thirteen. The food balls formed more posteriorly are considerably smaller than those formed by the first few anterior segments.

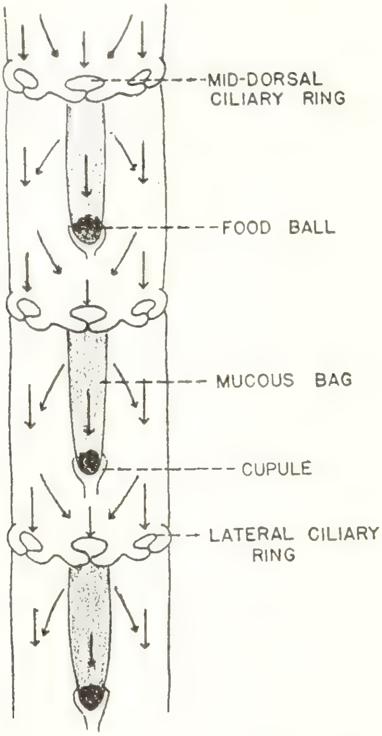


FIGURE 4. Dorsal view of three segments of middle body region showing the position of mucous bags and the formation of food balls. Arrows indicate the direction of water currents.

When a food ball of adequate size has been attained, secretion of mucous film is halted, and the bag is detached from the notopodial ring and completely rolled up by the cupule. The food ball is then carried forward along the mid-dorsal ciliary groove. On reaching an anterior cupule the food ball may be added to the food ball being formed there, or more frequently, the forward moving food ball forces the evacuation of the food ball in that cupule. The food balls thus tend to be carried anteriorly as an irregular string. When the food ball passes through a ciliary ring, which, it will be remembered, is driving water in the opposite direction, the two notopodia separate slightly and are inclined somewhat anteriorly.

As soon as the food ball passes, the notopodia return to their normal position. The entire cycle of movement takes place within a second or less. The membranelles were never observed to cease beating nor to reverse their beat.

Eventually the food balls reach the anterior end of the dorsal groove, which terminates just in front of the convex lower lip flanking the posterior side of the mouth (Fig. 1, B). They are then driven by the general surface ciliation over the lip into the mouth.

The time required for complete formation of a food ball was not measured. When worms were fed a suspension of carmine particles or stained Pablum, food balls were formed and passed anteriorly in less than a minute. But this is certainly far more rapid than would occur under natural conditions where detritus is fine and less concentrated.

The presence of a food bag tends to change the pressure of the water current passing through the dorsal ciliary ring as compared to the lateral rings. This results in the production of compensating currents crossing between the three main streams (Fig. 4). Thus, some of the water passing through the lateral rings at one level passes over and enters the middle ring at the next segment, bringing with it a new source of detritus to be removed by the food bag at this level. Eventually, almost all of the water passing posteriorly through the tube is strained through a mucous bag. This apparently occurs by the level of the first twelve or so segments and would account for the decreasing size of the food balls and absence of mucous bag formation more posteriorly.

Although feeding most commonly occurs with the head directed upward within the tube, worms were observed on occasion to feed while in a head downward position.

The principal function of the palps, as will be described later, is egestion and maintenance of an unobstructed tube. But the palps were occasionally observed to act as secondary or accessory feeding organs. Small masses of detritus were carried down the palps and into the mouth within the large ciliated gutter of the palp.

Water circulation and egestion

Much of the body of *Spiochaetopterus* is clothed in fine cilia, but it is the membranelles of the notopodial rings that create the water current upon which the life of the worm depends. The membranelles were never observed to cease beating nor were they ever observed to reverse their beat. Their beating creates a continual water current passing through the tube. Although feeding is restricted to the first twelve or so notopodial rings, all of the notopodial rings are involved in the production of the water current. When the worm is directed upward, the current enters the mouth of the tube at the surface of the substratum; when the worm is directed downward, the current enters the buried lower end of the tube, seeping in from the surrounding mud and sand.

The continual passage of water through the tube functions not only as a feeding current, as has already been described, but also as a respiratory current and a current for the removal of waste. *Spiochaetopterus* is devoid of gills, as might be expected in an animal of such small size. Respiratory exchange occurs across the general body surface.

The long delicate palps of *Spiochaetopterus* are highly effective organs for the removal of egested waste and any over-large masses of detritus brought in by the water current. Each palp is provided with a small ciliated groove which runs just lateral to the broad, deep, gutter. Cilia within the groove beat anteriorly toward the palp tip, *i.e.*, in the opposite direction from that of the water current produced by the membranelles. Any large object or large mass of detritus carried into the tube by the water current becomes entangled in mucus produced by the palps. The undesired material is then carried anteriorly along the ciliated groove of one of the palps. If the worm is below the upper end of the tube, as is often the case, it moves upward in the tube until the tips of the palps project from the tube opening. The detritus mass being carried along the palpal gutter is now ejected from the tip of the palp and falls to the surface of the surrounding substratum.

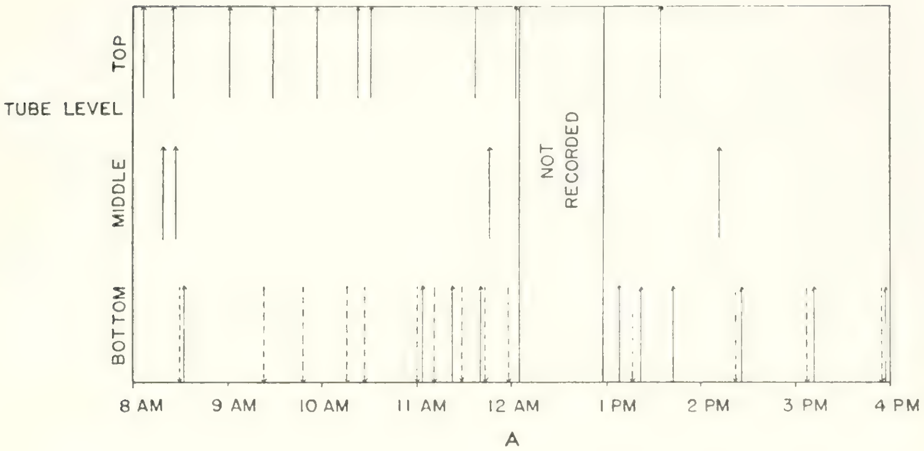
The efficiency of the palps in preventing clogging of the tube was especially evident in feeding experiments using a suspension of carmine particles or dyed Pablum. When a drop of suspension was released at the opening of the tube, a large amount of the material was immediately drawn into the tube. However, as soon as the suspended material reached the level of the palps, the larger particles were quickly collected by these structures and moved on the palpal groove back toward the exterior. The worm rapidly moves upward in the tube during this process. Only the finest of the suspended particles passes by the palps and is collected by the mucous bags.

The palps also function in the removal of feces. Egested wastes are released from the anus as elongated fecal pellets, shaped like a grain of rice and about twice the size of a food ball. On leaving the anus the pellets are picked up by the ciliated mid-dorsal groove, which runs the entire length of the body, beginning just in front of the anal opening. This is the same groove which transports the food balls, and in the mid-region of the body the fecal pellets pass through the notopodial ciliary rings in the same manner as a food ball. On reaching the anterior end of the dorsal groove, the pellets are transferred to the palpal groove for ejection to the exterior. With every release of a fecal pellet by the anus, and several may be released at one time, the worm moves toward the top of the tube to permit the palps to eject the pellets from the tube opening. How fecal pellets are differentiated from food balls is not clear, for only occasionally is a fecal pellet ingested. Perhaps the difference in shape or size is a factor.

Patterns of activity

Worms placed in capillary tubes could be kept in good condition and observed for weeks. The easy adjustment of these animals to glass tubes made it possible to observe in some detail the activity patterns of their tubicolous existence. The only limitation to these observations was the fatigue of the observer who had to constantly watch the worms both with the unaided eye and through a dissecting microscope. From a number of such observations, two seven-hour records (Fig. 5) are presented as being fairly representative. The data provided by these observations should in large part be applicable to worms living under normal conditions in natural tubes.

A number of facts regarding the activity patterns of *Spiochaetopterus* are indicated by these records. The worms are by no means stationary inhabitants of



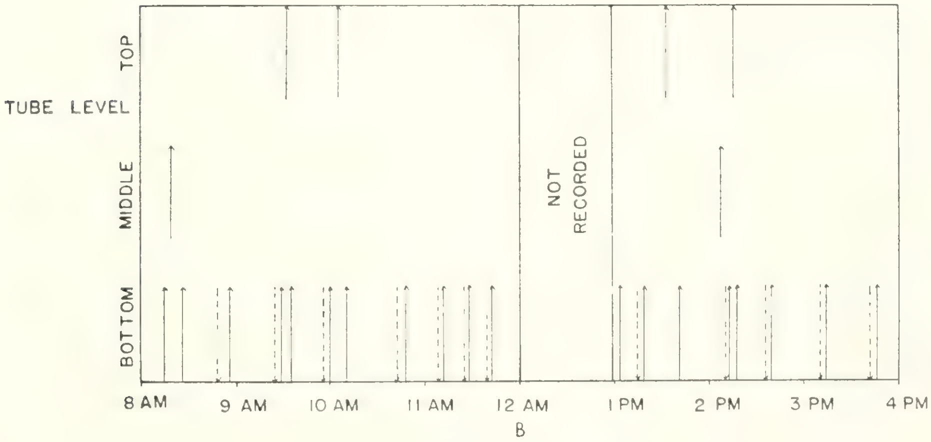


FIGURE 5. Records of the activity patterns of two different worms over a seven-hour period. Each arrow represents a position assumed by the worm and the time that the position was first assumed. The interval of time spent in that position is indicated by the space between two arrows. Solid arrows represent a head-upward position; dashed arrows represent a head-downward position. The three vertical levels represent levels occupied by the worm within the tube. Worm A spent a total of 66% of the time recorded at the bottom of the tube. Worm B spent over 90% of the time recorded at the bottom of the tube.

their tubes, but continually shift position within the tube. As many as eight or nine changes in position occur within an hour. Yet the pattern of movement is very irregular; it is not cyclic nor predictable.

Although a worm frequently inverts itself and is capable of remaining upside down for a long period of time, it most frequently assumes an upright position. This is not surprising in view of the structure of the tube, the means by which a water current is produced, and the mechanism of feeding and egestion. Inversion a lengthy sequence with tube-building activity at the lower end of the tube. It [illegible] be noticed that [illegible] is usually [illegible].

Resting is the usual activity when the worm is stationary, and the worm can [illegible] at any level within the tube. However, this activity is more commonly carried out at the bottom of the tube. All worms observed spent the greater part of the [illegible] at the bottom of the tube. [illegible] The worm would then return to the bottom.

Although there were [illegible]

DISCUSSION

[illegible] a number of [illegible] The genus *Chaetopterus* contains the largest members of the family, and the tube [illegible]

In *Chaetopterus* [illegible]

The [illegible] U-shaped tube of *Chaetopterus* keeps the tube flushed clean [illegible]

[illegible]

Telepsavus, *Phyllochaetopterus*, and *Mesochaetopterus*—all possess long palps and significantly all construct more or less straight tubes with the lower end buried in the substratum. Only *Chaetopterus* has short palps, and only members of this genus construct U-shaped tubes with separate surface openings for the entrance and exit of the water current. It is probable that the presence of little palps in *Chaetopterus* is a secondary condition.

The composition of the tube is parchment-like in *Chaetopterus*, *Mesochaetopterus*, and *Ranzanides*, and is cornified, or chitin-like, in *Spiochaetopterus*, *Phyllochaetopterus* and *Telepsavus* (Fauvel, 1927). However, in both *Chaetopterus* and *Spiochaetopterus* the glands for the secretion of the tube are located on the ventral side of the anterior section of the body and the process of secretion occurs in a somewhat similar manner. This is very likely true of the other members of the family as well.

All of the Chaetopteridae possess the enlarged, highly modified 4th neuropodial setae described for *Spiochaetopterus*, and as in *Spiochaetopterus* it is probable that these setae function in all members of the family to cut open the side of the tube wall. Enders (1909) described the opening of the side of the tube by *Chaetopterus* but did not explain the mechanism. It is very likely that the function is performed by the 4th setae.

Confirmation of some of these speculations regarding tube-building and feeding must await a study of other members of this very unique family of polychaetes. The author hopes to undertake this task in the near future.

SUMMARY

1. *Spiochaetopterus oculatus* secretes a small, cornified, annulated tube approximately 0.75 mm. in diameter and eight or more centimeters in length. The tube is constructed vertically in intertidal or subtidal sand and mud with only a few millimeters of the upper end of the tube projecting above the surface of the substratum.

2. The tube is secreted by epidermal glands occupying almost the entire ventral surface of the anterior body region.

3. One annular half-cylinder of tube is secreted at a time, the length of the cylinder corresponding to a section between annulations. The wall is gradually thickened with additional secretions.

4. Temporary, transverse, lamina-like partitions are constructed in the lower part of the tube. The partitions are perforated by several openings.

5. An entire new lower region of tube may be constructed as a branch of the old tube. The lower part of the old tube is then sealed off.

6. In constructing such a branch, the old tube wall is cut open with one or a pair of greatly enlarged blade-like setae of the fourth segment. These setae are also used to remove the temporary tube partitions.

7. The water current through the tube is produced by the beating of [illegible] lamellae which line three ring-like openings formed by the foliaceous notopodia of each segment in the middle region of the body.

8. The mid-dorsal ring of each of the first 12 or so foliaceous notopodia secretes a mucous bag which strains out fine detritus and plankton from the water current passing through the tube. The mucous bag is [illegible] and rolled up as a

food ball by a cupule located behind each ring. The food ball is then passed forward to the mouth along a ciliated mid-dorsal groove which runs anteriorly from the posterior tip of the body.

9. The greatly elongated palps function chiefly to remove feces and also to remove over-large masses of detritus which enter the tube with the water current.

LITERATURE CITED

Enders, H. E., 1908. Observations on the formation and enlargement of the tubes of the marine annelid, *Chaetopterus variopedatus*. *Proc. Indiana Acad. Sci.*, **1907**: 128–135.

Enders, H. E., 1909. A study of the life history and habits of *Chaetopterus variopedatus*. *J. Morph.*, **20**: 479–532.

Fauvel, P., 1927. Polychètes sedentaires. *Faune de France*, **16**: 1–494.

MacGinitie, G. E., 1939. The method of feeding of *Chaetopterus*. *Biol. Bull.*, **77**: 115–118.

MacGinitie, G. E., and N. MacGinitie, 1949. Natural History of Marine Animals. Pp. 1–473. McGraw-Hill Book Co., Inc., New York.

SEQUENTIAL INDUCTION OF THE PRESUMPTIVE EPIDERMIS OF THE RANA PIPIENS GASTRULA[1]

LESTER G. BARTH AND LUCENA J. BARTH

Marine Biological Laboratory, Woods Hole, Massachusetts 02543

A study of the effects of inductors applied for various lengths of time and at various concentrations has led us to the concept of sequential induction (Barth and Barth, 1963). The competent ectoderm responds to an inductor by first forming radial nerve. Further application of the inductor results in the differentiation of spreading nerve; and with still further treatment the cells which would have formed spreading nerve are induced to form pigment cells. Two more cell types in the sequence are astrocytes and neuroglia cells.

The experiments reported in this paper were designed to test the hypothesis of sequential induction by the sequential action of two different inductors. Specifically, if we first induce presumptive epidermal cells to become determined as nerve cells, will a second inductor then induce these determined nerve cells to become pigment cells?

We also asked the question: is induction at any one step in the sequence reversible? To answer this question we first induced the cells to become determined as pigment cells and then applied a strong neural inductor to see if it could reverse the induced pigment cells back to nerve cells.

Finally, we designed some experiments to test whether or not the sequence of inductions was a necessary sequence. Can pigment cells be induced without first inducing nerve cells?

During the course of the experiments it became apparent that we were obtaining a new cell type between spreading nerve and pigment cells. These cells possess the slate gray pigment granules characteristic of pigment cells, but these granules are distributed throughout the cytoplasm rather than forming a ring of pigment. The cells remain in clumps, in contrast to the later behavior of pigment ring cells. Finally, they have thick, prominent cell membranes. Thus, with the addition of this new cell type the sequence of inductions becomes epidermis, to radial nerve, to spreading nerve, to slate gray epithelium, to pigment cells, to astrocytes to neuroglia.

EXPERIMENTAL METHODS

Explants of the presumptive epidermis are dissected out of 6 gastrulae at Stage 11 Shumway (1940) unless otherwise stated. Six explants are treated with Versene (EDTA) to loosen the bond between the outer pigmented layer of epidermis and the inner layer. The outer pigmented layer is removed and discarded. The inner layer then is divided into about 25 small aggregates of cells.

---

[1] Supported by a grant from the Department of Health, Education and Welfare, HD 00701-01, to the Marine Biological Laboratory, and by GM 03322-13 to Columbia University.

These 150 aggregates then are transferred to solutions containing the inductors and finally to small stender dishes containing our standard salt solution, which also contains globulin from serum (Bios Laboratories, Inc., 17 W. 60th St., New York 23). An adequate substitute for Bios globulin has been found to be lyophilized calf serum (Nutritional Biochemicals Corp.). The method is the same as described by Barth and Barth (1959) except that we do not use agar to coat our operating dishes. This is because agar was found to be a neural inductor (Barth and Barth, 1963).

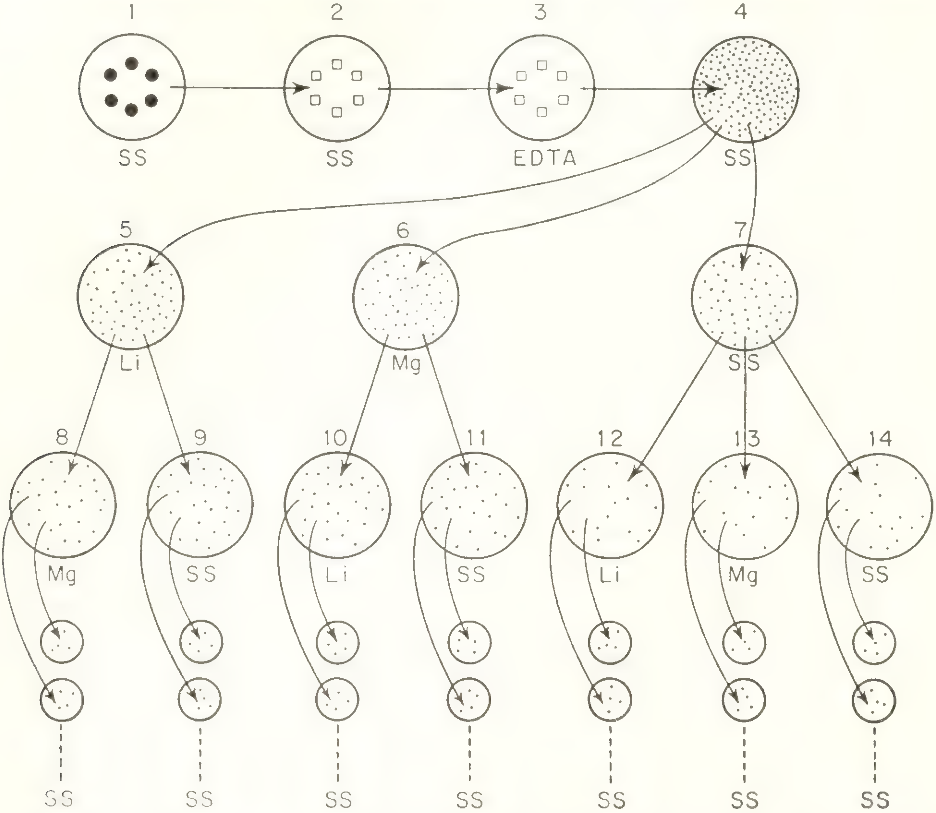


FIGURE 1. Diagram of a typical experiment involving the use of two inductors in sequence. Vitelline membranes are removed in dish 1 containing standard solution (SS). Composition of this solution is given in Barth and Barth (1959). Explants are dissected out in dish 2. Versene-treatment in dish 3 loosens bond between outer pigmented layer of epidermis and inner layer of cells. In dish 4 outer pigment layer is removed and discarded, and explants are teased into aggregates. Subsequent transfers of aggregates, all made by means of Spemann pipette, are described in the text. Abbreviations used are as follows: Li: lithium chloride; Mg: $MgSO_4 \cdot 7\ H_2O$.

A diagram of a typical experiment involving the use of two inductors in sequence is presented in Figure 1. The experiment diagrammed gives the following information.

1. Transfers from 4 to 7 to 14 serve as a control for differentiation of untreated epidermal cells. Such cells usually form a sheet of epithelium with ciliated patches or a mass of free-swimming ciliated cells. The control is necessary because sometimes for unknown reasons a small amount of nerve develops in controls.

2. Transfers from 4 to 6 to 10 give the sequential effect of Mg followed by Li, which results in extensive pigment cells. Two controls for this effect are (a) 4 to 6 to 11, which shows that Mg alone does not induce pigment cells, and (b) 4 to 7 to 12, which gives the effect of Li without previous treatment with Mg—usually a few scattered pigment cells or none at all.

3. Transfers from 4 to 5 to 8 show the reversibility of the Li induction by Mg. Usually no pigment cells differentiate. Transfers 4 to 5 to 9 give the results of Li alone, where some pigment cells differentiate. Sequential transfers from 4 to 7 to 13 show that Mg is still able to induce nerve cells.

Several preliminary experiments of the type outlined above showed that Mg would induce nerve, which was then induced to pigment cells by Li. However, Li, which induced pigment cells, followed by Mg resulted in no pigment cells but only nerve cell differentiation. More detailed experiments in which the times of treatment and the concentrations of two inductors were varied are given in the experimental results.

EXPERIMENTAL RESULTS

Sequential action of lithium chloride after pretreatment with magnesium sulfate

In these experiments we attempted to achieve a situation by which a minimal or subminimal exposure to lithium chloride would result in a few pigment cells or none at all. Some of the aggregates were first treated with magnesium sulfate, which would induce the cells to become nerve. Other aggregates were exposed to the standard solution for the same length of time as those which were exposed to magnesium sulfate. Other controls consisted of treatment with magnesium sulfate without subsequent treatment with lithium chloride; and controls for the standard solution, *i.e.*, no treatment with either inductor but the same number of transfers at the same times.

Table I records the sequential action of magnesium sulfate followed by lithium chloride. In this and the succeeding tables the table headings are to be interpreted as follows. Stage no.: Shumway (1940); conc.: concentration of added substances in milligrams per milliliter of standard solution; time: time in hours during which the aggregates are exposed to the substances indicated; types of cellular differentiation: as described in Barth and Barth (1963) and Barth and Barth (1962).

Experiments 1–5 show that lithium chloride alone induces only a few pigment cells while most of the cells form nerve or epithelium. If the explant were first treated with magnesium sulfate for 2.5 to 3.5 hours, which is sufficient for the induction of nerve, lithium chloride induces most of the cells to differentiate into pigment cells and spreading nerve. Thus, magnesium sulfate, which is unable to induce pigment cells by itself, can induce nerve cells, which are then further induced by lithium chloride to become pigment cells.

Experiments 7 and 8 show sequential action with regard to the type of nerve induced. It is seen that magnesium sulfate induces radial nerve. If it is followed by increasing exposures to lithium chloride the radial nerve is induced to the spreading nerve pattern of differentiation. In these experiments no pigment cells are induced because the treatments begin at early stage 11 and the cells are not competent for pigment cell induction at this stage with the times of exposure to lithium used in the experiments (Barth, 1964).

TABLE I

Sequential action of magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$) *and lithium chloride* (*LiCl*)

Exp. no.	Stage no.	Mg		Li		Number of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	6.0	3.0	2.0	2.0	75	Extensive pigment cells, spreading nerve
	11	0.0	3.0	2.0	2.0	75	Epithelium, few pigment cells, little nerve
2	11	6.0	2.5	2.0	3.0	75	Extensive pigment cells, nerve
	11	0.0	2.5	2.0	3.0	75	Epithelium, few pigment cells, little nerve
3	11	6.0	3.0	2.0	2.0	75	Extensive pigment cells, little nerve
	11	0.0	3.0	2.0	2.0	75	Epithelium, few pigment cells, little nerve
4	11	6.0	3.0	2.0	2.0	50	Extensive pigment cells, spreading nerve
	11	0.0	3.0	2.0	2.0	50	Epithelium, few pigment cells, some nerve
	11	6.0	3.0	0.0	2.0	25	Nerve
	11	0.0	3.0	0.0	2,0	25	Epithelium
5	11	6.0	3.5	2.0	2.0	40	Extensive pigment cells, spreading nerve
	11	0.0	3.5	2.0	2.0	35	Nerve, few pigment cells, ciliated cells
6	11 –	6.0	2.5	2.0	2.0	40	Slate gray epithelium, some nerve
	11 –	0.0	2.5	2.0	2.0	35	Spreading nerve, some slate gray epithelium
7	11 –	6.0	2.3	3.0	0.5	15	Radial nerve
	11 –	0.0	2.3	3.0	0.5	15	Epithelium
	11 –	6.0	2.3	3.0	1.0	15	Spreading nerve, some radial nerve
	11 –	0.0	2.3	3.0	1.0	15	Some nerve, some epithelium
	11 –	6.0	2.3	3.0	1.5	15	All spreading nerve
	11 –	0.0	2.3	3.0	1.5	15	Radial nerve, some spreading nerve
8	11 –	6.0	2.0	4.3	0.5	25	Spreading nerve
	11 –	0.0	2.0	4.3	0.5	25	Radial nerve
	11 –	6.0	2.0	4.3	1.0	25	Spreading nerve
	11 –	0.0	2.0	4.3	1.0	25	Radial nerve

Experiment 6 gives results intermediate between those of 1–5 and 7 and 8. Early stage 11 is used and the cells are not competent yet for pigment cell induction. Lithium chloride induces mostly spreading nerve and a few slate gray cells of a type obtained many times with lithium chloride when applied to early stage 11 cells. However, after pretreatment of the preparations with magnesium sulfate, lithium chloride induces many more slate gray cells and less nerve appears. These slate gray cells resemble those of the adrenal medulla in staining properties, but have not been positively identified as such. In any case these cells form a step in the sequence between spreading nerve and pigment cells and they possess the same type of slate gray granules as do pigment cells.

Sequential action of calcium chloride followed by lithium chloride

Table II, experiment 1, records the data supporting the conclusion that a pretreatment with calcium chloride permits lithium chloride at minimal exposure to induce extensive pigment cells. Experiment 2 shows that increasing exposure to lithium chloride induces the nerve induced by calcium chloride to differentiate into spreading nerve and finally slate gray epithelium. In experiment 3, when a longer exposure to lithium chloride results in extensive pigment cells, pretreatment with calcium chloride gave not only extensive pigment cells but also some astrocytes.

TABLE II

Sequential action of $CaCl_2 \cdot 2\ H_2O$ *and LiCl*

Exp. no.	Stage no.	Ca		Li		Number of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	2.5	2.1	4.2	1.1	75	Extensive pigment cells, some spreading nerve
	11	0.0	2.1	4.2	1.1	75	Epithelium, some pigment cells, little nerve
2	11 –	2.5	2.6	4.2	0.3	25	Spreading nerve, radial nerve
	11 –	0.0	2.6	4.2	0.3	25	Epithelium, little nerve
	11 –	2.5	2.6	4.2	0.7	25	Spreading nerve, little radial nerve
	11 –	0.0	2.6	4.2	0.7	25	Radial nerve, epithelium
	11 –	2.5	2.6	4.2	1.0	25	Spreading nerve, slate gray epithelium
	11 –	0.0	2.6	4.2	1.0	25	Radial nerve, spreading nerve, few slate gray cells
3	11	2.5	3.2	3.0	2.0	60	Extensive pigment cells, some astrocytes, some nerve
	11	0.0	3.2	3.0	2.0	60	Extensive pigment cells, some nerve

From all the experiments in Table II we conclude that lithium chloride appears to carry on the sequence of inductions from radial nerve to spreading nerve to slate gray cells to pigment cells and finally to astrocytes.

Combination of the action of an inductor followed by culture in a low concentration of lithium chloride

Continuous culture in low concentrations of lithium chloride in the standard solution brings about the induction of astrocytes and neuroglia cells. At a concentration of 0.40 mg./ml. astrocytes form; at 0.47 to 0.65 mg./ml. neuroglia differentiate. If we first induce the cells to spreading nerve or pigment cells and then culture in low concentrations of lithium chloride what cell types would we obtain?

TABLE III

Combination of the actions of an inductor and a low concentration of LiCl in the culture solution. Li:LiCl; Mg: $MgSO_4 \cdot 7\ H_2O$; Ca: $CaCl_2 \cdot 2\ H_2O$.

Exp. no.	Stage no.	Treatment		Culture	No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.			
1	11	3.0 Li	2.0	.00 Li	50	Extensive pigment cells
	11	3.0 Li	2.0	.15 Li	50	Extensive pigment cells, some astrocytes
	11	3.0 Li	2.0	.30 Li	50	Extensive neuroglia cells
2	10+	6.0 Mg	3.0	.10 Li	35	Spreading nerve, radial nerve
	10+	0.0 Mg	3.0	.10 Li	35	Ciliated masses, mucus
	10+	6.0 Mg	3.0	.30 Li	48	Some large, unidentifiable cells
	10+	0.0 Mg	3.0	.30 Li	48	Ciliated masses, mucus
3	11−	6.0 Mg	4.0	.10 Li	35	Spreading nerve
	11−	0.0 Mg	4.0	.10 Li	35	Ciliated epithelium, voluminous mucus
	11−	6.0 Mg	4.0	.30 Li	40	Large, unidentifiable cells
	11−	0.0 Mg	4.0	.30 Li	40	Ciliated epithelium, voluminous mucus
4	11	6.0 Mg	3.0	.20 Li	25	Loose cells, some ciliated masses
	11	6.0 Mg	3.0	.00 Li	25	Spreading nerve, radial nerve
5	11	6.0 Mg	3.0	.10 Li	35	Spreading nerve, radial nerve
	11	6.0 Mg	3.0	.05 Li	40	Spreading nerve, radial nerve
	11	6.0 Mg	3.0	.00 Li	35	Spreading nerve, radial nerve
	11	0.0 Mg	3.0	.10 Li	30	Ciliated epithelium
6 and 7	11	2.5 Ca	3.0	.10 Li	35	Spreading nerve
	11	0.0 Ca	3.0	.10 Li	35	Ciliated masses
	11	2.5 Ca	3.0	.30 Li	40	Loose cells, no differentiation
	11	0.0 Ca	3.0	.30 Li	40	Ciliated masses, voluminous mucus

In Table III, experiment 1, lithium chloride is first used to induce pigment cells and then the aggregates are cultured in 0.15 and 0.30 mg./ml. of lithium chloride. Astrocytes appear at 0.15 mg./ml. and neuroglia at 0.30 mg./ml. Lithium chloride in these low concentrations has little effect on aggregates, as seen from experiment 3. Pretreatment with lithium chloride to induce pigment cells permits the further induction to astrocytes and neuroglia.

The remainder of the table, experiments 2–7, records attempts to obtain inductions with low concentrations of lithium chloride after inducing the cells with magnesium sulfate or calcium chloride. It is clear that either calcium chloride or magnesium sulfate will induce the spreading nerve stage, but that subsequent treatment with 0.10 mg./ml. of lithium chloride has no effect. Subsequent treatment with 0.30 mg./ml. of lithium chloride has a deleterious effect with many loose, dead cells and only a few large, unidentifiable cells remaining alive. These results, when compared with those of experiment 1, lead us to conclude that the induction of pigment cells is a necessary step toward the induction of astrocytes and neuroglia cells. Since neither calcium chloride nor magnesium sulfate is able to induce pigment cells, further induction by low concentrations of lithium chloride is blocked.

Reversibility of the induction: induction of pigment cells by lithium chloride followed by a neural inductor

In Table IV experiments 1–4 record data which are interpreted to mean that either the induction of pigment cells is reversible or that pigment cells cannot differentiate after treatment with magnesium sulfate. Experiment 3 in particular shows that lithium chloride alone induces pigment cells and magnesium sulfate alone induces nerve cells. When the two are applied in sequence with lithium chloride first, only nerve cells are induced.

In experiment 5 lithium chloride induced slate gray epithelium and, when further treated with magnesium sulfate, the cells formed only spreading nerve.

TABLE IV

Reversibility of the induction of pigment cells Li:$LiCl$; Mg: $MgSO_4 \cdot 7\ H_2O$

Exp. no.	Stage no.	Li		Mg		No. of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11	3.0	2.0	6.0	3.3	75	Spreading nerve, few slate gray cells
	11	3.0	2.0	0.0	3.3	75	Extensive pigment cells, nerve
2	11	3.0	1.5	6.0	2.0	50	Spreading nerve
	11	3.0	1.5	0.0	2.0	50	Few pigment cells, spreading nerve
3	11	3.0	2.0	6.0	3.0	40	Nerve
	11	3.0	2.0	0.0	3.0	40	Pigment cells, nerve
	11	0.0	2.0	6.0	3.0	35	Nerve
	11	0.0	2.0	0.0	3.0	35	Epithelium
4	11−	3.0	3.5	6.0	2.8	40	Spreading nerve
	11−	3.0	3.5	0.0	2.8	40	Few pigment ring cells, spreading nerve
5	11−	3.0	2.5	6.0	3.0	40	Spreading nerve
	11−	3.0	2.5	0.0	3.0	35	Nerve, slate gray epithelium
	11−	0.0	2.5	6.0	3.0	40	Nerve
	11−	0.0	2.5	0.0	3.0	35	Epithelium
6	11	4.0	2.0	7.0	3.0	60	Spreading nerve, slate gray epithelium
	11	4.0	2.0	0.0	3.0	60	Pigment cells, nerve
7	11	4.0	2.0	7.0	3.0	75	Spreading nerve, slate gray epithelium
	11	4.0	2.0	0.0	3.0	75	Pigment cells, nerve
8	11+	3.0	2.0	6.0	3.5	75	Extensive pigment cells, nerve
	11+	3.0	2.0	0.0	3.5	75	Extensive pigment cells, epithelium
9	11+	2.0	3.0	6.0	2.5	70	Extensive pigment cells, nerve
	11+	2.0	3.0	0.0	2.5	70	Extensive pigment cells, nerve

In experiments 6 and 7 [illegible] sodium chloride induced pigment cells; magnesium sulfate favored the induction of [illegible] epithelium.

The last two experiments, 8 and 9, were performed to distinguish between reversibility of induction of pigment cells and failure of pigment cells to differentiate. By using a later stage of development (stage 11) when extensive pigment cells are induced by a two- to three-hour period of exposure to lithium. Subsequent treatment with magnesium sulfate has no effect on the differentiation of the

TABLE IV

[illegible]

[illegible]	[illegible]	Treatment		[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]			
[illegible]	[illegible]	[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Spreading nerve
	[illegible]	[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Pigment cells, spreading nerve
[illegible]	[illegible]	[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Spreading nerve
	[illegible]	[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Pigment cells, spreading nerve
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Nerve, [illegible] epithelium
		[illegible]	[illegible]	[illegible]	[illegible]	Pigment cells, nerve
[illegible]		[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Nerve
		[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Pigment cells, nerve
		[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Nerve
		[illegible]	[illegible]	[illegible] $MgSO_4$	[illegible]	Epithelium
[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	Nerve
		[illegible]	[illegible]	[illegible]	[illegible]	Pigment cells, nerve
		[illegible]	[illegible]	[illegible]	[illegible]	Epithelium
		[illegible]	[illegible]	[illegible]	[illegible]	Pigment cells, nerve, epithelium
[illegible]			[illegible]	[illegible]	[illegible]	Nerve
				[illegible]	[illegible]	Nerve and pigment cells
				[illegible]	[illegible]	Nerve
				[illegible]	[illegible]	Nerve
			[illegible]	[illegible]	[illegible]	Numerous pigment cells
			[illegible]	[illegible]	[illegible]	Nerve
			[illegible]	[illegible]	[illegible]	Few pigment cells, nerve
				[illegible]	[illegible]	[illegible] pigment cells, [illegible]
				[illegible]	[illegible]	Nerve

pigment cells (Table IV). In experiment 8 the [illegible] magnesium sulfate.

[illegible]

[illegible]

used to induce pigment cells and then the aggregates were cultured subsequently in a neural inductor. Experiments 1–4 show reversibility of induction as a result of culture in magnesium sulfate, while experiments 5 and 6 give the data for reversibility due to culture in sodium bicarbonate. As can be seen in experiment 6 a few pigment cells are able to differentiate in the presence of the neural inductor, sodium bicarbonate, and this fact reinforces the interpretation that the neural inductor reverses induction of but does not interfere with the differentiation of, pigment cells.

Finally, we have a little data on the action of calcium chloride on reversibility of induction. Table VI shows that this compound in a concentration of 2.5 mg/ml, applied for two hours after induction of pigment cells by lithium chloride, changes pigment cells to slate gray epithelium or changes slate gray epithelium to nerve. If, on the other hand, the aggregates are cultured continuously in a low concentration of calcium chloride after induction by lithium chloride, as in experiments 3 and 4, extensive pigment cell differentiation can occur.

TABLE VI

Reversibility of induction of pigment cells by LiCl by culture in $CaCl_2$

Expt. no.	Stage no.	LiCl		$CaCl_2$		No. of aggregates	Types of cellular differentiation
		Conc.	Time hrs.	Conc.	Time hrs.		
1	11—	4.3	3.0	2.5	2.0	15	Nerve
	11—	4.3	3.0	0.0	2.0	11	Slate gray epithelium, nerve
2	10	4.3	3.0	2.5	2.0	12	Slate gray epithelium, nerve
	11	4.3	3.0	0.0	2.0	11	Pigment cells, nerve
3	11	4.4	3.0	0.25	—	15	Extensive pigment cells, nerve
	10	4.4	3.0	0.1	—	15	Few pigment cells, nerve
4	10—	4.5	3.0	0.25	—	10	Pigment cells, nerve

Simultaneous action of two inductors

As a matter of empirical interest we wanted to see what sort of induction would occur when two inductors were permitted to act at the same time. Since they do act synergistically in one sequence and antagonistically in the reverse sequence it would be difficult to predict what would happen when they act simultaneously. In the design of the experiment two difficulties arose: (1) the choice of the relative concentration of the two inductors; and (2) the total ionic strength, which in combination is much higher than when applied singly to explants. To some extent these problems can be met by using sodium bicarbonate as the second inductor, since it induces nerve cells in relatively low concentrations. Experiments 1 and 2 in Table VII summarize the data we have on the action of lithium chloride and sodium bicarbonate in combination. While lithium chloride alone induces extensive pigment cells in most cases when combined with sodium bicarbonate, few or no pigment cells differentiate. Similar results are obtained with calcium chloride and magnesium sulfate in experiment 3, 5, 6, 7, 8, 9. On the other hand, experiments 4 and 10 would suggest that a more extensive set of experiments, involving different concentrations of the two inductors, might show a synergistic action. These two experiments involved lower concentrations of

TABLE VII

Simultaneous action of two inductors

Li: $LiCl$; *Bi*: $NaHCO_3$; *Ca*: $CaCl_2 \cdot 2\,H_2O$; *Mg*: $MgSO_4 \cdot 7\,H_2O$

Exp. no.	Stage no.	Treatment			Number of aggregates	Types of cellular differentiation
		Conc.	Hrs.	pH		
1	11	2.0 Li + 1.5 Bi	2	9.0	25	Spreading nerve
	11	2.0 Li + 0.75 Bi	2	8.8	25	Spreading nerve
	11	2.0 Li	2	8.0	35	Nerve, pigment cells
	11	2.0 Li + 1.5 Bi	4	9.0	25	Nerve, few pigment cells
	11	2.0 Li + 0.75 Bi	4	8.8	25	Nerve, few pigment cells
	11	2.0 Li	4	8.0	35	Extensive pigment cells, little nerve
	11	2.0 Li + 1.5 Bi	6	9.0	25	Nerve, few pigment cells
	11	2.0 Li + 0.75 Bi	6	8.8	25	Nerve, few pigment cells
	11	2.0 Li	6	8.0	35	Extensive pigment cells, little nerve
2	11	2.0 Li + 1.0 Bi	4	8.8	75	Spreading nerve
	11	2.0 Li + 1.0 Bi	7	8.8	75	Spreading nerve
	11	2.0 Li	4	8.0	35	Extensive pigment cells, nerve
3	11	2.0 Li + 2.0 Ca	3		60	Nerve, rare pigment cells
	11	2.0 Li	3		60	Extensive pigment cells, little nerve
4	11	0.5 Li + 1.5 Mg	7		25	Epithelium, few pigment cells, nerve
	11	0.5 Li	7		25	Ciliated masses, mucus
	11	1.5 Mg	7		25	Epithelium, ciliated masses
5	11	4.0 Li + 6.0 Mg	2		50	Unknown cell types
6	11	2.0 Li + 4.0 Mg	3		75	Spreading nerve, short nerve
7	11	2.0 Li + 3.0 Mg	2.0		40	Nerve, few pigment cells
	11	2.0 Li	2.0		35	Extensive pigment cells
	11	2.0 Li + 3.0 Mg	4.0		40	Nerve, few pigment cells
	11	2.0 Li	4.0		35	Extensive pigment cells
8	11	2.0 Li + 3.0 Mg	2.0		40	Nerve, slate gray epithelium
	11	2.0 Li	2.0		35	Nerve, pigment cells, slate gray epithelium
	11	2.0 Li + 3.0 Mg	3.0		40	Nerve, pigment cells
	11	2.0 Li	3.0		35	Extensive pigment cells, nerve
9	11	2.0 Li + 3.0 Mg	2.5		60	Nerve, slate gray epithelium
	11	2.0 Li	2.5		60	Nerve, some pigment cells
10	11	1.0 Li + 3.0 Mg	3.7		75	Spreading nerve, pigment cells
	11	1.0 Li	3.7		75	Radial nerve, rare pigment cells

lithium chloride combined with magnesium sulfate applied for a longer period of time. Note that while neither lithium chloride nor magnesium sulfate induced nerve or pigment cells, the combination induced some nerve cells and a few pigment cells. Clearly no final conclusions can be drawn from the data in Table VII.

Is the sequence of inductions a necessary one?

From a theoretical point of view it is important to know whether in the sequence of inductions the steps are so related that one depends upon another, or whether a specific inductor could induce one cell type without first inducing the cell type preceding. More specifically, is it possible to induce pigment cells without first inducing radial nerve and then spreading nerve? This question arose from speculation that the sequence of inductions might be almost entirely the result of a sequence in competence of the presumptive epidermis.

We first determined the change in competence with time of the presumptive epidermis to form pigment cells when treated with a specific concentration of lithium chloride for a specific time (3.0 mg./ml. for two hrs.). These results are published in abstract form (Barth, 1964). The peak of competence was found to be between stages 11 plus and 12 minus. We then treated presumptive epidermis at these stages with lithium chloride for different lengths of time. The results are shown in Table VIII.

TABLE VIII

Sequential induction by lithium chloride in relation to competence

Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
	Conc.	Hrs.		
11+	3.0 Li	0.5	25	Epithelium, little nerve
11+	3.0 Li	1.0	25	Spreading nerve, few pigment cells
11+	3.0 Li	1.7	25	Extensive pigment cells, nerve
12−	3.0 Li	0.5	25	Epithelium, little nerve
12−	3.0 Li	1.0	25	Nerve, pigment cells
12−	3.0 Li	1.5	25	Extensive pigment cells, nerve

At both stages lithium chloride induces extensive pigment cells but as the duration of treatment is shortened nerve is induced instead of pigment cells. Thus, at the peak of competence for induction of pigment cells, lithium first induces nerve cells which then become further induced to pigment cells.

DISCUSSION

The process of induction in a sequence involves at least three basic phenomena. First, a cell type is determined and will reproduce itself during cell division. Second, the induction becomes irreversible, and thus the cell, having been induced, is inhibited in its capacity for differentiation into those cell types which precede it in the sequence. Third, the induction makes possible further induction, *i.e.,* the next step in the sequence of inductions. Thus, in the induction of a nerve cell from the presumptive epidermis we need the activation of a self-duplicating system, which is best provided by DNA. We also need an inhibitor to prevent the induced cell from becoming an epidermal cell. And in addition, the induction of a nerve cell makes possible the further induction of a pigment cell.

In the normal course of events the presumptive epidermal cell undergoes cell division and differentiates. Thus, the DNAs for epidermal cell differentiation are active. When the cell is induced not only must the DNAs for nerve cells be activated, but those for epidermis must be inhibited. Finally, after the DNAs for nerve cells are activated, and only after this induction, we find that pigment cells may be induced. Thus, the induction of nerve cell must result in the competence for pigment cell induction.

The most direct approach to the problem outlined above is to assume that in the early determination of a cell by induction at least three DNAs are activated as a cistron. One DNA results in the synthesis of a specific protein which determines the differentiation of the cell. Another DNA results in the formation of a protein which masks the preceding DNAs in the sequence of inductions. A third DNA would direct the synthesis of a protein which would confer competence for the next step in the sequence. This latter competence might simply be a partial unmasking of the DNA in a cistron for the next cell type in the sequence so that an inductor might be able to complete the unmasking.

The action of inorganic ions as inductors

Of the known effects of lithium chloride on cells, the increase in viscosity of proteins (Ranzi and Citterio, 1957) is the most directly related to any possible action upon nucleoproteins. A change in structure of the histones by lithium chloride may possibly free the sites of action of the DNA and start the sequence of inductions. Nuclear histones, as regulators of gene activity, have been discussed recently by several investigators, including Busch *et al.* (1963), Bloch (1963), Horn (1962), and B. C. Moore (1963). Moore suggests that the genes are closely associated with histone and are prevented from synthesizing messenger RNAs before gastrulation. After gastrulation the association between histone and DNA changes and messenger RNAs are synthesized.

Using the above hypothesis, two mechanisms are needed for controlling the association between histone and DNA. During gastrulation there is a change in competence with time, which is independent of inductors. This is an intracellular control of gene action. Second, the inductors act on the competent cells and new genes must be activated. This is an extracellular control of gene action.

The first mechanism need not necessarily involve the actual removal of histone with the resultant activation of the genes, but rather make the histone susceptible to the action of an inductor. The repeated cell divisions during gastrulation, with the resultant exposure of the chromosomes to the action of cathepsins, may possibly remove most of the histone but leave enough to block one or more active sites on the DNA molecule. Then an inductor by uniting with histone may remove the last barrier to gene action.

Another possible way of reducing the histone is by repeated cell division. If DNA replicates faster than histone is synthesized, then the ratio of histone to DNA would be lowered. Extending this process beyond gastrulation, a time might arrive when the genes for the differentiation of epidermis were activated by removal of histone. No extracellular inductor is necessary for the determination of ciliated cells of the epidermis.

Quite clearly the main difficulty in the hypothesis of the control of gene action by histones is lack of evidence for the specificity necessary to activate one set of genes in one group of cells and another set in a second group of cells. Obviously some histones would need to remain in association with DNA while others would be removed. In the case of sequential induction the histone associated with neural genes would need to be more susceptible to the action of inductors than the histone combined with pigment cell genes. The concept of sequential induction might be of help in reducing the numbers of histones to the numbers of sequential inductions. Thus, perhaps one kind of histone is associated with those genes involved in ectodermal differentiation, another in mesodermal differentiation and a third in endodermal differentiation. In any one sequence of inductions the amount of histone might possibly determine the susceptibility of any one set of genes to the action of an inductor.

Relation of sequential induction to the organizer

In the case of the organizer phenomenon we have a situation by which chordamesoderm induces the presumptive neural plate to form the definitive neural plate, and at the same time becomes definitive notochord and mesoderm. The median portion of the neural plate differentiates into motor neurons, while the lateral regions form neural crests which differentiate into sensory neurons, sympathetic neurons, pigment cells, adrenal medulla and other cell types. The presumptive chordamesoderm has competence for differentiation into neural plate and the presumptive neural plate has competence for differentiation into chordamesoderm. Thus, one basic question arises: Why doesn't the presumptive chordamesoderm differentiate into neural plate since it has both the competence and the inductor for neural plate? Next, we have to account for the induction of forebrain by the prechordal plate and spinal cord by the chordamesoderm. In addition, we would like to have an explanation for the medio-lateral differences in the neural plate, since these arise shortly after the time of induction (Corner, personal communication).

One basic assumption will be made. The living organizer produces an inductor which induces different cell types at different concentrations. The highest concentration of the inductor will then be in the chordamesoderm itself and will induce these cells to become notochord and mesoderm. As gastrulation occurs, the next highest concentration of the inductor will be in the posterior presumptive neural plate, because this region is in contact with the inductor for the longest period of time, and also the chordamesoderm has greater mass per unit area in this region. The anterior presumptive neural plate will have the lowest concentration of the inductor for two reasons. First, it is in contact with the prechordal plate for only a short time before competence is lost, and second, because the prechordal plate is very thin and the concentration of the inductor must be correspondingly low. Mangold (1933) commented on the very weak inductive capacity of the prechordal plate.

Within the chordamesoderm during gastrulation a situation develops whereby the notochord is a thin strip of tissue applied to the median region of the presumptive neural plate, while the mesoderm becomes concentrated in a much thicker layer under the lateral regions of the neural plate. Thus, the concentration of the inductor will be higher in the lateral regions as compared with the median region.

Therefore, we visualize a situation by which a single inductor, at a concentration C_n induces chordamesoderm; at a concentration of C_{n-1} induces neural crest; at a lesser concentration, C_{n-2}, spinal cord; at C_{n-3}, forebrain. What evidence do we have to support this hypothesis?

With regard to induction of forebrain, most of the evidence supports a weak inductor. (1) In *Ambystoma maculatum* no external inductor at all is necessary for induction of forebrain (Barth, 1941; Holtfreter, 1944). (2) The spinocaudal inductor, when partly denatured, induces forebrain (Yamada, 1958). (3) The mesodermal inductor, when heated, loses its inductive properties in the order of spinocaudal, hindbrain, forebrain, to no induction (Yamada, 1958). (4) Mangold (1933) tested the anterior region of the roof of the archenteron in the blastocoel of an early gastrula and found it to have weak inductive capacity as compared with more posterior regions.

The induction of the medio-lateral regionality in the neural plate can be imitated to some extent by lithium chloride. With a low concentration of, or short exposure to, lithium chloride, the presumptive epidermis forms motor neurons, as evidenced by their action to stimulate muscle to contract (Barth and Barth, 1959). Higher concentrations of lithium chloride or longer exposure time results in neural crest, as evidenced by pigment cell differentiation and a type of nerve which is similar to sympathetic nerve.

Finally, have we any single inductor which actually does induce mesoderm, notochord, and neural tissue? Masui (1960) has obtained excellent induction of notochord, muscle, pronephros, nerve, pigment cells and mesenchyme by the use of lithium chloride. In our own experiments we were not so successful as Masui, but we did obtain at least two types of nerve, pigment cells, mesenchyme, but only sporadically notochord and muscle. Finally, it is clear that one substance is able to induce differentiation of many cell types, and we believe that this constitutes good evidence for a single inductor in the organizer region of the gastrula.

A number of objections may be made against the hypothesis of a single inductor present in the organizer region and acting by inducing various cell types at various concentrations. Among these objections we will consider two. First, how can we reconcile the fact that one half of the organizer region will induce a bilaterally symmetrical neural plate? The differences in concentration of a single inductor would be expected to be radically altered after bisection of the organizer, and an asymmetrical neural plate would result.

Second, the fact that a non-organizer region containing no inductor may be placed in the organizer region with no change in the neural plate induced requires an explanation. Small transplants of presumptive epidermis placed in the organizer region regulate, undergo gastrulation and become inductors. How can the differences in concentration of the inductor required by the hypothesis be maintained under these circumstances?

The two criticisms chosen above may be leveled at most hypotheses of organizer action and are not particular criticisms of the single inductor hypothesis. Indeed, it would appear to be even more difficult to interpret the experiments on division of the organizer and substitution of the organizer if the hypothesis of a mosaic of inductors is offered.

SUMMARY

1. A sequence of inductions has been obtained with a single inductor applied to the presumptive epidermis in various concentrations and for varying lengths of time.

2. A neural inductor, such as magnesium sulfate or calcium chloride, will induce a part of the sequence such that a subminimal exposure to lithium chloride then is able to induce pigment cells.

3. After lithium chloride is used to induce pigment cells, a strong neural inductor such as sodium bicarbonate, magnesium sulfate or calcium chloride applied immediately after lithium treatment may reverse the induction and nerve cell differentiation will result.

4. The sequence of inductions is a necessary one, as evidenced by the fact that at the peak of competence for pigment cell induction, lithium chloride first induces nerve cells and then pigment cells.

LITERATURE CITED

BARTH, L. G., 1941. Neural differentiation without organizer. *J. Exp. Zool.,* **87**: 371–384.

BARTH, LUCENA JAEGER, 1964. Control of cellular differentiation by inorganic ions in relation to competence. *Amer. Zool.,* **4**: 317.

BARTH, L. G., AND L. J. BARTH, 1959. Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. Exp. Morphol.,* **7**: 210–222.

BARTH, L. G., AND L. J. BARTH, 1962. Further investigations of the differentiation *in vitro* of presumptive epidermis cells of the *Rana pipiens* gastrula. *J. Morphol.,* **110**: 347–373.

BARTH, L. G., AND L. J. BARTH, 1963. The relation between intensity of inductor and type of cellular differentiation of *Rana pipiens* presumptive epidermis. *Biol. Bull.,* **124**: 125–140.

BLOCH, D. P., 1963. Genetic implications of histone behavior. *J. Cell. Comp. Physiol.,* **62**: 87–94.

BUSCH, H., W. J. STEELE, L. S. HNILICA, C. W. TAYLOR, AND H. MAVIOGLU, 1963. Biochemistry of histones and the cell cycle. *J. Cell. Comp. Physiol.,* **62**: 95–110.

HOLTFRETER, J., 1944. Neural differentiation of ectoderm through exposure to saline solution. *J. Exp. Zool.,* **95**: 307–340.

HORN, E. C., 1962. Extranuclear histone in the amphibian oocyte. *Proc. Nat. Acad. Sci.,* **48**: 257–265.

MANGOLD, O., 1933. Über die Induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwiss.,* **43**: 761–766.

MASUI, Y., 1960. Alteration of the differentiation of gastrula ectoderm under influence of lithium chloride. *Mem. Kōnan Univ., Sci. Ser., No. 4, Art. 17,* 79–102.

MOORE, BETTY C., 1963. Histones and differentiation. *Proc. Nat. Acad. Sci.,* **50**: 1018–1026.

RANZI, S., AND P. CITTERIO, 1957. Proteine e determinazione embrionale nella *Rana,* nel riccio di mare e negli incroci dei rospi. *Acta Embryol. et Morph. Exp.,* **1**: 78–98.

SHUMWAY, W., 1940. Stages in the normal development of *Rana pipiens. Anat. Rec.,* **78**: 139–147.

YAMADA, T, 1958. Embryonic induction. *In:* The Chemical Basis of Development, pp. 217–238. Ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md.

PROPERTIES OF THE DACTYL CHEMORECEPTORS OF CANCER ANTENNARIUS STIMPSON AND C. PRODUCTUS RANDALL[1]

JAMES CASE

Department of Biological Sciences, University of California, Santa Barbara, California

Behavioral and electrophysiological observations have indicated the presence of chemoreceptors particularly sensitive to amino acids on the dactylopodites of the green crab, *Carcinus* (= *Carcinides*) *maenas* (Case, Gwilliam and Hanson, 1960; Case and Gwilliam, 1961). Although sufficient information regarding chemoreception in various arthropods previously had accumulated to render those observations not unexpected (Luther, 1930; Hodgson, 1958; Barber, 1961), recently Laverack (1963) has been unable to detect responses to amino acids in an electrophysiological examination of the dactyl innervation of the European *Carcinus*. Here we attempt to resolve the problems thus raised concerning the reality of dactyl amino acid receptors in crabs by demonstrating their presence in two species of yet another brachyuran genus and by considering their chemical sensitivity in some detail.

Preliminary reports of this investigation have appeared (Case and Gwilliam, 1963; Case, 1964).

MATERIALS AND METHODS

The experimental material consisted primarily of dactylopodites of any pereiopod of mature *Cancer productus* Randall and *C. antennarius* Stimpson. No variations in sensory responses could be attributed to differences between species or sexes or among legs. Dactyls were prepared for recording, after limb transsection between propodite and carpopodite, by dissection of musculature and articulations of the dactylopodite-propodite joint, leaving undisturbed the centrally placed nerve. The distal centimeter of the dactyl was then pushed through a small hole in a rubber sheet which formed one end of a sea water-filled chamber into which the nerve floated as the propodite was pulled away. Nerve bundles were subdivided and arranged for monopolar recording at the water surface on a bare silver electrode leading to a P5C Grass A.C. amplifier. Amplified signals were led to an audio monitor and Tektronix 502 oscilloscope. Provisions were available for tape recording and for signal integration, the latter by means of a modified Offner myograph integrator (1.0 sec. time constant) and oscillograph.

Test materials were made up in sea water, neutralized except where specified, and applied usually as approximately 0.03-ml. single drops to the moist dactyl tip in air. Washing with at least 15 drops of sea water followed the stimulus drop within a few seconds except when persistent stimulant effects were under study. Test and wash solutions and the preparations were all held at 16° to 18° C.

---

[1] Supported by U.S.P.H.S. Grant NB-04372 and Faculty Research Funds from the University of California.

Study of the receptors of any dactyl began with selection of a subdivided bundle containing only a few chemoreceptive units, all giving substantial responses to single drops of 0.05 *M* glycine and with endings suitably placed for stimulation in a restricted area on the dactyl tip proximal to the heavily chitinized cap. Response magnitude was ordinarily determined from the first 1.5 second of the integrated response and expressed as an activity ratio (AR), the ratio of the experimental response to the reaction of that same preparation to 0.05 *M* glycine. Since the standard glycine response was determined every few tests, expressing stimulatory effectiveness in this way served to compensate for temporal variation in sensitivity on the part of the same preparation, for variation in sensitivity and number of active units among preparations, and for the contribution of non-chemoreceptive units to the response.

RESULTS AND SPECIFIC COMMENTS

1. *Discrimination between mechano- and chemoreceptors.* Mechanoreceptor activity commonly occurs along with chemoreceptor responses within subdivided nerve bundles, as in Figure 1A, although not necessarily (Fig. 5). Continued subdivision of bundles carrying mixed activity frequently results in isolation of fascicles in which chemoreceptor units are dominant, the situation in Figure 1B. Note, however, that this investigation offers no conclusive evidence concerning the possibility that chemoreceptor units may also be excited by physiologically reason-

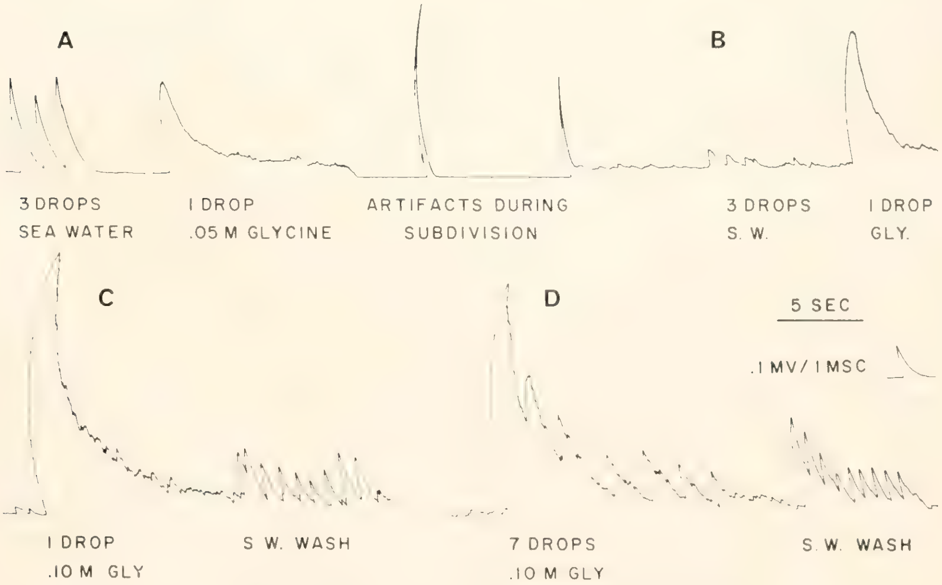


FIGURE 1. Integrated responses from dactyl innervation. A and B demonstrate improvement in recording of chemoreceptor activity in course of subdivision of nerve. C and D, records from another dactyl showing similar rates of response decay when dactyl is stimulated with either one or several drops of stimulant. Notches in D indicate mechanoreceptive responses to successive drops of test solution. *C. productus.*

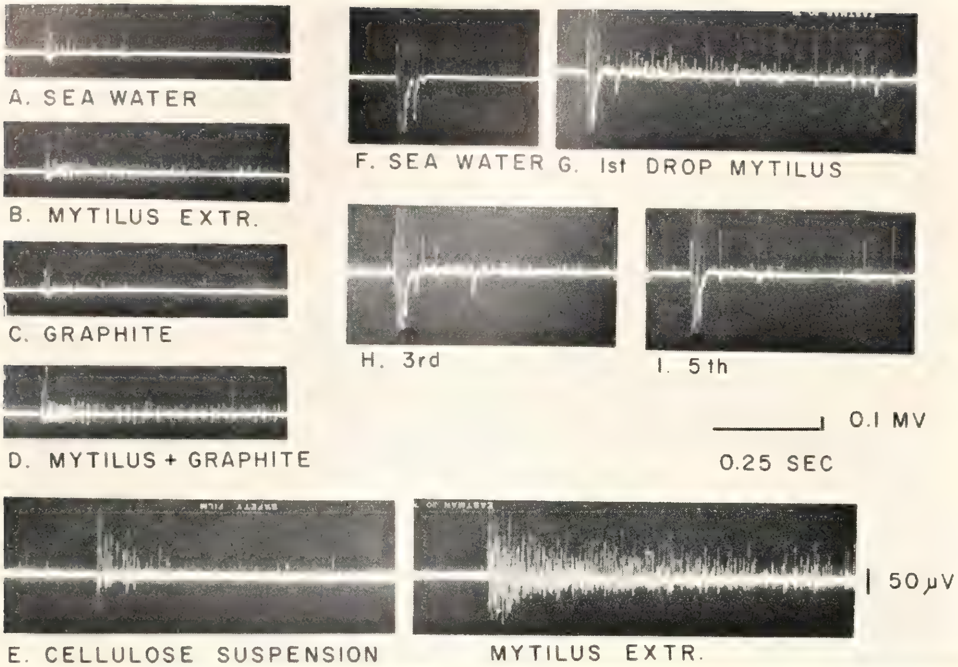


FIGURE 2. Comparison of responses to mechanical and chemical stimulation of dactyl. See text for details. *C. productus.*

able mechanical stimuli. That a form of synergy may occur between chemical and mechanical stimuli is frequently suggested by situations in which the first drops of sea water wash after chemical stimulation elicit considerably larger responses than later drops in the wash series, as illustrated in the wash responses of Figure 1D.

When both kinds of sensory activity appear in the same fascicle, they are readily distinguished by simple experiments of the type demonstrated in Figure 2, in this instance specifically concerned with the extent to which a sea water extract of *Mytilus* tissue stimulates chemically and mechanically. Three different fascicles are represented, all obviously containing mechanically sensitive components as shown in Figure 2A, E, F. The responses in Figure 2A, B are obviously unlike in that the extract causes prolonged activity in small units which are not activated by the sea water control. Since a microscopic examination shows that filtrate is by no means particle-free, a thick suspension of graphite[2] particles, similar in size to those found in the extract, is applied, with the minimal effect apparent in Figure 2C. Subsequent application of the extract without washing away the graphite shows that the previous treatment has not inactivated the receptors (Fig. 2D). The even larger particles of a cellulose suspension produce only minimal responses as compared with the extract in a test on another fascicle (Fig. 2E, H).

[2] "Prodag," Acheson Colloids Co. semi-colloidal graphite, used after prolonged washing.

TABLE I

Summary of dactyl preparations responsive to 0.05 M glycine

	Responsive	Non-responsive	Per cent responsive
C. productus (n = 28)			
2d pereiopod	15	6	
3d pereiopod	9	4	
4th pereiopod	11	2	
5th pereiopod	11	5	
Unspecified	10	0	
	56	17	77 (73 preparations)
C. antennarius (n = 30)			
2d pereiopod	34	5	
3d pereiopod	26	3	
4th pereiopod	25	1	
5th pereiopod	14	1	
	99	10	91 (109 preparations)
Totals, both species	155	27	85 (182 preparations)

Another means of differentiating between mechano- and chemoreceptor units is illustrated in the remainder of Figure 2. A pure mechanoreceptive response to a drop of sea water occurs in Figure 2F. The remaining illustrations in the series, Figure 2G, H, I, are examples of responses to a long series of drops of *Mytilus* extract. It is clear that the chemoreceptor element of the response

TABLE II

Examples of distribution of chemo- (CR) and mechanoreceptor (MR) activity in dactylus and propus innervation

Fascicle (in order of testing)	*C. productus* 5th. pereiopod				*C. antennarius* 4th. pereiopod			
	Dactylus		Propus		Dactylus		Propus	
	CR	MR	CR	MR	CR	MR	CR	MR
1	+	+	0	0	0	0	+	+
2	+	+	+	+	0	0	0	0
3	0	0	0	0	0	0	0	+
4	+	+	0	+	0	0	0	+
5	+	+	0	0	+	0	0	+
6	0	+	0	0	0	0	0	+
7	+	+	0	0	+	+	+	+
8	0	0	0	0	+	+	0	+
9	0	0	0	0	+	+	+	0
10	0	+	0	+	+	+	0	0
11	+	+	0	0	+	+	0	0
Total +	6	8	1	3	6	5	3	7

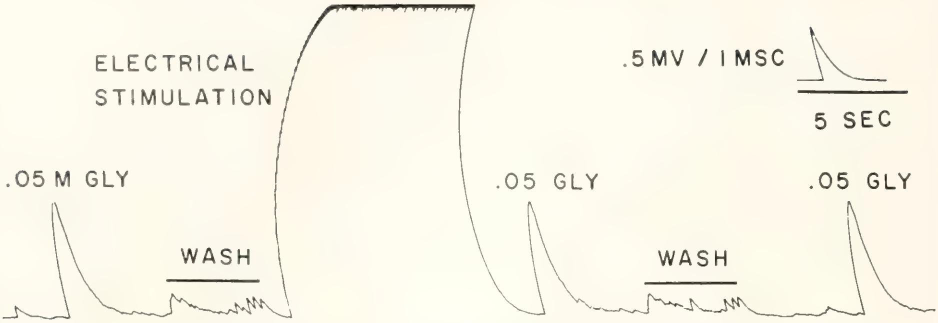


FIGURE 3. Demonstration that decay of chemoreceptor response is not due to fatigue of electrically excitable receptor elements. See text for details. *C. antennarius.*

adapts considerably more readily than the mechanoreceptive element. This aspect of chemoreceptor activity is discussed in more detail below.

2. Prevalence and distribution of chemosensory units. The percentage of preparations responsive to 0.05 *M* glycine in the entire experimental series is presented in Table I, from which it is evident that amino acid-sensitive units are common on all pereiopods of both *C. productus* and *C. antennarius.* The frequency of successful preparations is probably unrealistically low in the case of *C. productus* since most failures to demonstrate chemoreceptors in that species involved only five crabs in which chemoreceptors were not evident in any limb.

Few data regarding chemosensitivity of chelipeds have been obtained because their size makes them awkward to prepare for recording. It can only be reported that both of two chelipeds of *C. antennarius* which were tested were responsive to 0.05 *M* glycine, the only substance tested. Chelipeds have been shown behaviorally to mediate feeding responses to several amino acids (Case and Gwilliam, 1961; 1963).

Undoubtedly chemoreceptors are not confined to the dactylopodites. This

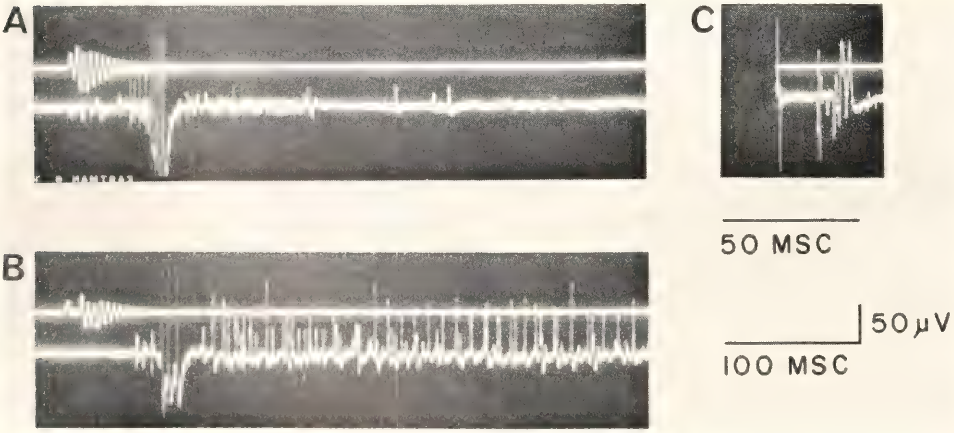


FIGURE 4. Illustration of method of determination of latency; 100-msc. calibration applies to A and B. See text for details. *C. antennarius.*

is demonstrated in Table II which summarizes the distribution of chemo- and mechanoreceptor activity in a number of randomly selected fascicles from the innervation of both dactylopodite and propodite. Obviously both limb segments are well supplied with chemoreceptor endings. No significance can be attached to the indication of a larger number of chemoreceptive units in the dactyl, since the experimental arrangement permitted exploration of little more than half the propodite surface, while nearly all the dactyl surface was exposed to testing.

Although more proximal limb segments have not been tested electrophysiologically, behavioral tests show that they do mediate feeding responses, as contrasted, for example, with the dorsal body surface (Case, unpublished observations).

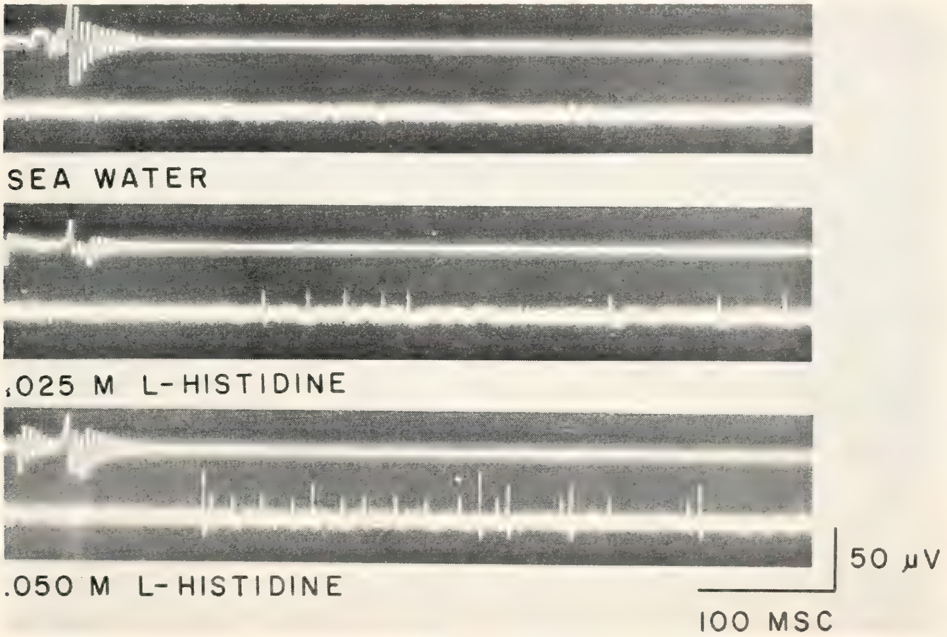


FIGURE 5. Responses of a pure chemoreceptor bundle to 0.05 *M* L-histidine, showing effect of concentration on threshold and latency of two units. Widest excursion of upper trace indicates arrival of stimulus drop. *C. antennarius.*

3. Temporal characteristics of chemoreceptor responses. The usual time course of responses to effective chemical stimulants consists of attainment of maximum activity within the first 0.2 second after stimulus application with a markedly slower decline to half maximum in one to two seconds. Activity subsequently approaches the resting level more slowly with no obvious suggestion of stabilizing above rest level. As far as approximately the initial 10 seconds of the response are concerned, this pattern, well illustrated in the integrated records of Figure 1A, B, cannot be the consequence of the technique of stimulation, namely application of single drops of test solutions. Thus, in Figure 1D the response curve in which seven drops of glycine are applied is virtually identical with the response in Figure 1C, produced by a single drop of glycine, save for mechanoreceptor responses.

Conceivably, rapid response decay can be attributed to effects upon specific chemoexcitatory processes leading to impulse generation. When, as in Figure 3, a small receptor population is excited electrically with an electrode pair placed nearby on the integument, all peripheral, electrically excitable elements of the impulse-generating sequence, except the specific chemosensory mechanism, are undoubtedly excited. Activation of the receptors in this way, to an extent far greater than the response to a standard glycine stimulus, has no evident effect upon an immediately ensuing response to chemical stimulation.

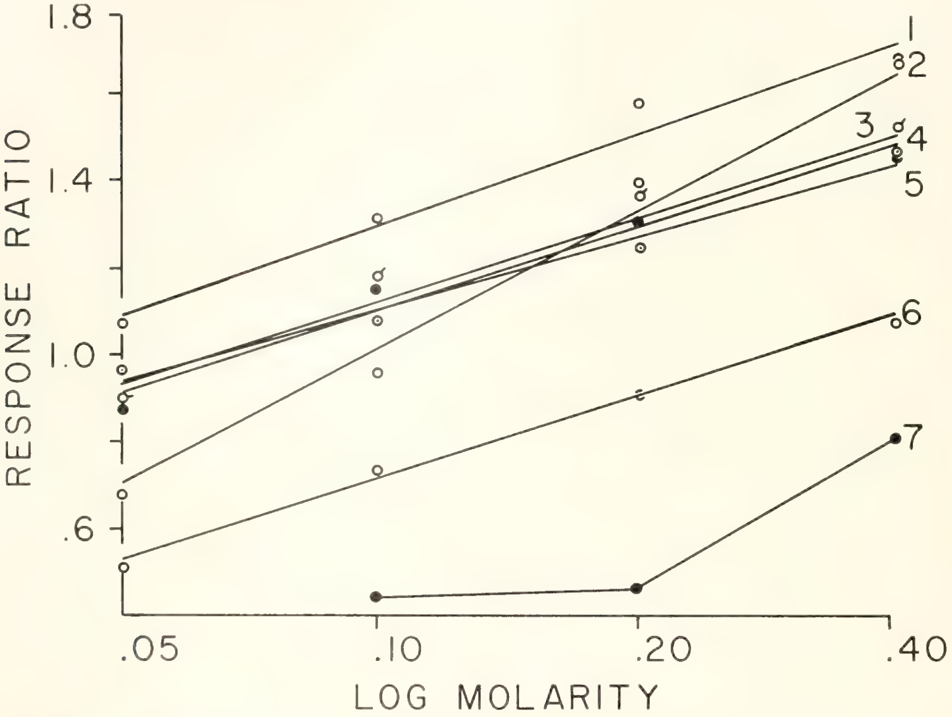


FIGURE 6. Concentration-activity relationships of various stimulants. 1, L-serine; 2, L-alanine; 3, glycine; 4, L-proline; 5, L-threonine; 6, glycyl-glycine; 7, trimethylamine. Plots 1–6 calculated by least squares. Five preparations tested at all concentrations of all compounds in ascending order of concentrations with two-minute washes between tests. Data not used in the tabulations of this paper. *C. antennarius.*

Somewhat different responses were observed more rarely. These were characterized by abrupt transition from a decay curve of the usual form to a much more gradual decline towards rest level. If fascicles behaving in this manner did arrive at an elevated steady-state, rest level was probably not exceeded by more than 10%. We have no clear idea at present concerning the significance of such responses. Their rarity and the fact that they resemble the obviously abnormal responses to high concentrations of certain chemical agents (see below) suggests

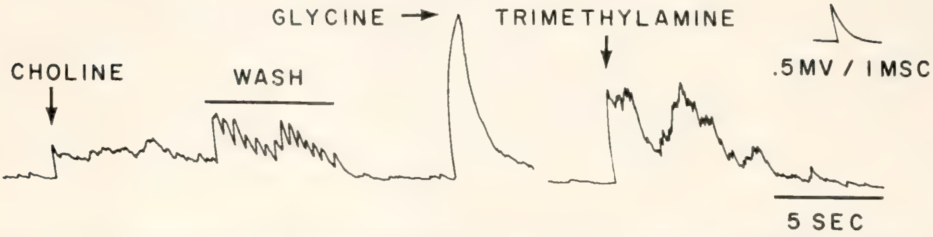


FIGURE 7. Comparison of responses to 0.40 *M* choline, 0.05 *M* glycine and 0.40 *M* trimethylamine. Approximately 5 seconds deleted from record between glycine and trimethylamine.

they are not typical. Consequently, preparations responding in this manner to 0.05 *M* glycine have not been used further in this investigation.

Response latency was determined approximately by means of a phonograph pick-up with a fine glass stylus positioned immediately above the dactyl in the path of the drop of stimulant, as illustrated in Figure 4, where response to a drop of sea water (Fig. 4A) appears in contrast with the response of the same units to 0.01 *M* L-glutamic acid (Fig. 4B). In each display initial deflection of the upper trace signals contact of the stimulus drop with the stylus, with the largest deflection when the drop leaves the stylus. The earliest mechanoreceptor activity in Figure 4A appears after 24 msc. while the earliest obvious chemoreceptor activity is seen after about 56 msc. in Figure 4B. Conduction velocities of 2.4 meters/sec. for chemoreceptor and 3.5 meters/sec. for mechanoreceptor axons were estimated for this preparation by measuring the rate of propagation over a 21-mm. length of the same bundle (Fig. 4C). The latencies for this preparation consequently become 9.8 msc. for the earliest mechanoreceptor and 35 msc. for the earliest chemoreceptor response to glutamic acid. Chemoreceptor latencies can obviously be

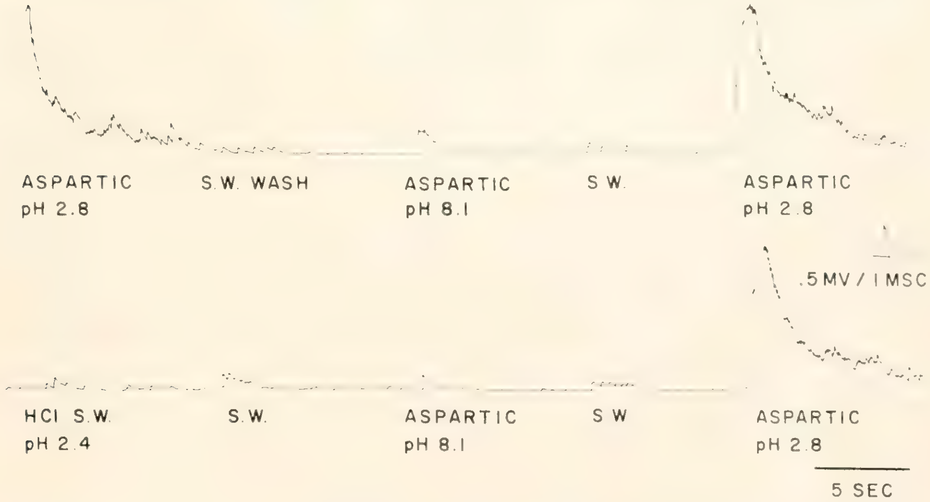


FIGURE 8. Influence of pH on response to 0.04 *M* D-aspartic acid. Upper record continuous with lower. *C. productus.*

expected to vary with the nature of the stimulant and with stimulant concentration, as illustrated in Figure 5 where the smallest of the three chemoreceptors responds earlier to a higher concentration of histidine.

4. *Response magnitude as a function of stimulant concentration.* Response magnitude of effective stimulants is linearly related to the logarithm of the concentration (Fig. 6). Six compounds, representative of the array of substances which appear to be effective stimulants, were tested in the range 0.05 to 0.04 *M*

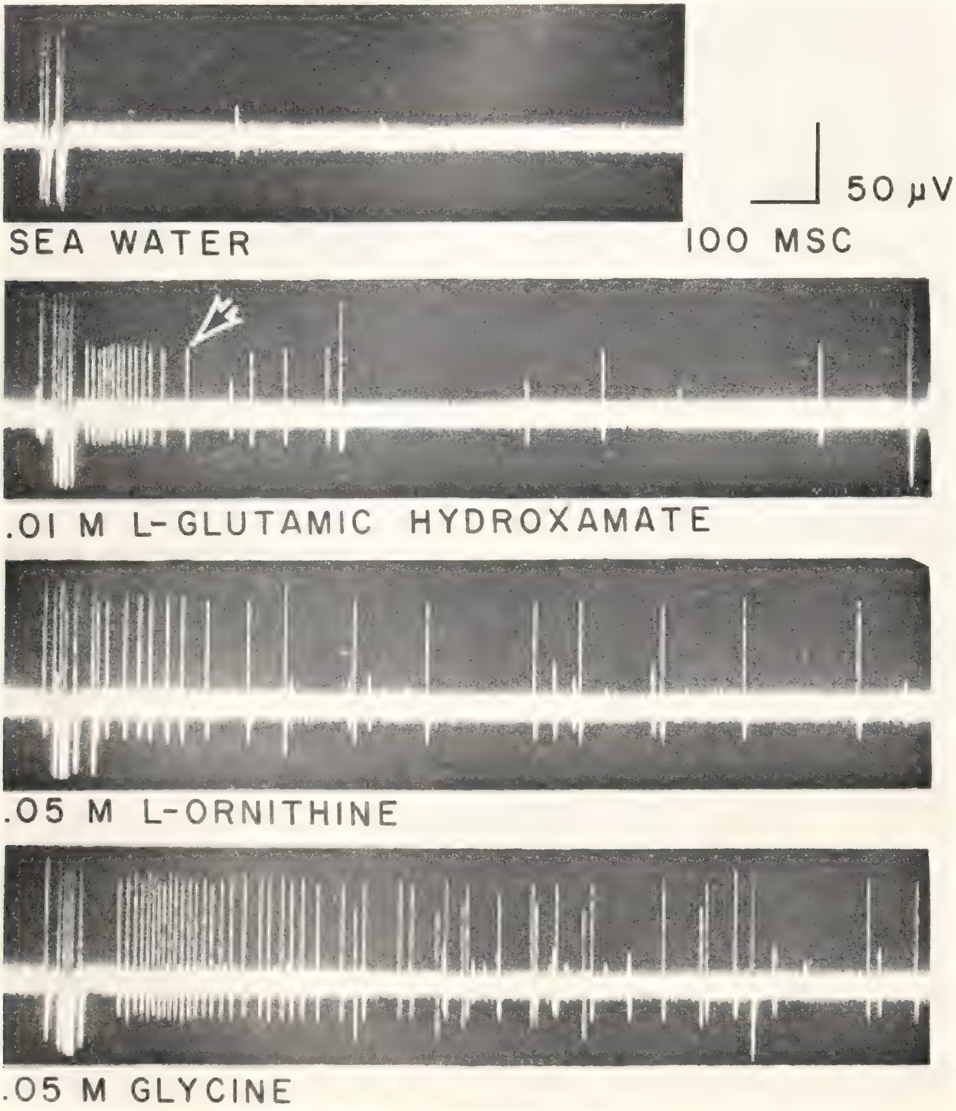


FIGURE 9. Multifiber preparation illustrating variation in chemical specificity. See text. *C. antennarius.*

TABLE III

Activity ratios of amino acids and related compounds. I. Aliphatic amino acids

Compound	Structure	AR*	SD**	N***
glycine	$CH_2(NH_2)CO_2H$	1.00 (arbitrary)		
N-carbamyl glycine	$(NH_2)CONHCH_2CO_2H$	0.25 ±	0.10	13
N,N-dimethyl glycine	$(CH_3)_2NCH_2CO_2H$	0.75	0.21	11
N-methyl glycine (sarcosine)	$CH_3NHCH_2CO_2H$	0.98	0.17	10
L-alanine	$CH_3CH(NH_2)CO_2H$	0.88	0.16	10
D-alanine	—	0.70	0.14	10
β-alanine	$NH_2CH_2CH_2CO_2H$	0.81	0.11	9
L-serine	$CH_2(OH)CH(NH_2)CO_2H$	1.13	0.23	9
taurine	$HO_3SCH_2CH_2(NH_2)$	1.41	0.26	10
cysteine	$CH_2SHCH(NH_2)CO_2H$	0.79	-	5
DL-α-amino-n-butyric acid	$CH_3CH_2CH(NH_2)CO_2H$	1.70	0.37	10
α-amino isobutyric acid	$(CH_3)_2C(NH_2)CO_2H$	0.38	0.14	9
DL-β-amino isobutyric acid	$(NH_2)CH_2CH(CH_3)CO_2H$	1.12	0.10	8
DL-β-amino butyric acid	$CH_3CH(NH_2)CH_2CO_2H$	0.92	0.06	9
γ-amino butyric acid	$NH_2CH_2(CH_2)_2CO_2H$	0.31	0.12	11
L-threonine	$CH_3CH(OH)CH(NH_2)CO_2H$	1.05	0.17	11
L-methionine	$CH_3SCHCH_2CH(NH_2)CO_2H$	0.78	0.13	10
α-methyl-DL-methionine	$CH_3SCHCH_2C(CH_3)(NH_2)CO_2H$	0.68	0.11	7
L-valine	$(CH_3)_2CHCH(NH_2)CO_2H$	1.00	0.19	9
D-valine	—	1.00	0.17	8
L-leucine	$(CH_3)_2CHCH_2CH(NH_2)CO_2H$	0.78	0.18	8
D-leucine	—	0.56	0.12	11
L-norleucine	$CH_3(CH_2)_3CHNH_2CO_2H$	0.61	0.11	9
L-isoleucine	$CH_3CH_2CH(CH_3)CH(NH_2)CO_2H$	0.73	0.21	11
D-isoleucine	—	0.54	0.15	9
L-lysine	$CH_2(NH_2)(CH_2)_3CH(NH_2)CO_2H$	0.37	0.08	10
ϵ-amino caproic acid	$(NH_2)CH_2(CH_2)_4CO_2H$	0.27	0.07	7
L-arginine	$(NH_2)C(NH)NHCH_2(CH_2)_2CH(NH_2)CO_2H$	0.39	0.09	11
α-ϵ-diaminopimelic acid	$HO_2CC(NH_2)(CH_2)_3CH(NH_2)CO_2H$	0.29	0.17	13

* Activity ratio; ** standard deviation; *** number of tests. Number of animals tested is approximately ½ this.

on *C. antennarius*. Of these, only L-alanine significantly differed in regression from the others ($P < 0.01$) while all six regressions of concentration *versus* response were significantly linear. Some compounds, such as trimethylamine and choline, do not appear to have a linear dose-response relationship in the same concentration range. As indicated in Figure 6, trimethylamine is essentially inactive until a concentration somewhat above 0.10 M is attained, whereupon response magnitude increases rapidly. An integrated record of response to 0.40 M trimethylamine is illustrated in Figure 7 in comparison with a standard glycine response. Trimethylamine responses are typically as shown, often bimodal and markedly different from the typical glycine response pattern. Choline, like trimethylamine, is ineffective except at high concentrations which produce a sustained, low intensity response.

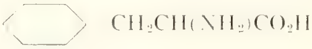
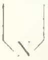
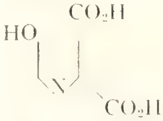
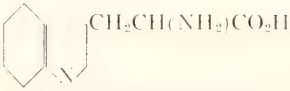
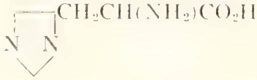
5. The effect of pH. The receptors are not excited either by acids such as hydrochloric or acetic at pH 2.0 or by sodium hydroxide at pH 9.0. Glycine and proline underwent no change in excitation efficiency over the same pH range.

In contrast to these observations L-aspartic acid is at least 10 times as active in acid solutions as near neutrality (Fig. 8). The speculation engendered by this observation, that the undissociated molecule might be the more excitatory, was not supported in the present experiments by the uniform activity of L-arginine in both alkaline and neutral solutions.

6. The adequate stimulus of the dactyl receptors. The observations of this section were obtained from *C. antennarius*.

TABLE IV

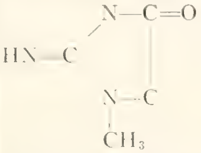
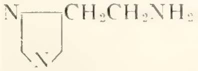
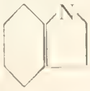
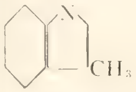
Activity ratios of amino acids and related compounds. II. Dicarboxylic Acids

Compound	Structure	AR	SD	N
L-aspartic acid	$HO_2CCH_2CH(NH_2)CO_2H$	0.29 ±	0.15	24
D-aspartic acid	—	0.93	0.13	20
DL-β-methyl aspartic acid	$HO_2CCH_2C(CH_3)(NH_2)CO_2H$	0.22	0.14	22
L-glutamic acid	$HO_2C(CH_2)_2CH(NH_2)CO_2H$	1.37	0.29	12
D-glutamic acid	—	0.51	0.19	10
N-carbamyl-L-glutamic acid	$HO_2C(CH_2)_2CH(NHCONH_2)CO_2H$	0.20	0.10	15
DL-N-methyl glutamic acid	$HO_2C(CH_2)_2CH(NHCH_3)CO_2H$	0.24	0.15	8
L-α-amino adipic acid	$HO_2C(CH_2)_3CH(NH_2)CO_2H$	0.58	0.20	7
	III. Aromatic and Heterocylic compounds			
L-phenylalanine		0.38	0.16	10
L-proline		1.00	0.17	14
hydroxy-L-proline		1.00	0.22	7
L-tryptophan		0.34	0.11	10
L-histidine		0.81	0.28	14
D-histidine		0.46	0.22	8

One aspect of the experimental method must be emphasized in regard to these experiments, namely that receptor populations have been examined, not single units. At this juncture it is impossible to determine how many receptor types, described in terms of response specificities, constitute the dactyl chemoreceptor population. While it is true that units conforming to our selection criterion of fast-adapting responses to 0.05 *M* glycine do respond in a uniform manner to many other chemical stimuli, there are certainly population variations in threshold and absolute specificity. To illustrate: D- and L-aspartic acids have never been tested without finding D-aspartic acid to be at least five times as active as its enantiomer (see below), an unexpectedly fine discrimination, but one evidently found in all members of the receptor population; yet threshold variations are common among members of this same population, as in Figure 5 where the two larger units are evidently less sensitive to L-histidine than the smaller one. Further, evidence of absolute specificity differences is common, as exemplified in Figure 9 where one unit is responsive to a glutamic acid derivative but not to L-ornithine and probably not to glycine. We believe, however, that these variations within the receptor population are minor enough to justify a survey of chemical specificity with multiple unit preparations selected according to the criteria described.

TABLE V

Activity ratios of amino acids and related compounds. IV. Amides, Amines, Miscellaneous

Compound	Structure	AR	SD	N
L-asparagine	$CONH_2CH_2C(NH_2)CO_2H$	0.70 ±	0.10	12
creatinine		0.37	0.18	16
L-glutamine	$(NH_2)OCH_2(CH_2)_2CH(NH_2)CO_2H$	0.61	0.12	7
ethanolamine	$HOCH_2CH_2NH_2$	0.41	0.08	7
cadavarine	$(NH_2)CH_2(CH_2)_3CH_2(NH_2)$	0.09	0.10	12
histamine		0.30	0.17	11
trimethylamine	$(CH_3)_3N$	0.40	0.09	12
glycine betaine	$(CH_3)_3NCH_2CO_2H$	1.06	0.10	9
choline	$(CH_3)_3N(OH)CHCH_2OH$	0.25	0.11	9
acetylcholine	$(CH_3)_3N(CH_2)_2OCOCH_3$	0.26	0.07	9
DL-carnitine	$(CH_3)_3NCH_2CH(OH)CH_2CO_2H$	0.33	0.10	13
glutaric acid	$HO_2C(CH_2)_3CO_2H$	0.35	0.18	20
α-ketoglutaric acid	$HO_2C(CH_2)_2COCO_2H$	0.25	0.19	13
indole		0.20	0.09	8
3-methyl indole		0.15	0.07	8
urea	$OC(NH_2)_2$	0.17	0.09	8

The principal results of the survey of chemical specificity are contained in Tables II, IV, V, and VI and Figure 7 with the following observations emergent:

The receptors are highly responsive to α-amino acids and certain related substances. The preponderance of evidence set forth in the tabulations shows the receptors are most responsive to α-amino and α-imino acids. Neither organic acids nor amines are conspicuously active (Tables III, IV and V). Indole, 3-methyl indole and trimethylamine, which might well appear in the dietary of these crabs, are similarly ineffective. The sugars, sucrose, glucose and trehalose, were non-stimulatory, as were all salts tested, namely KCl, NH_4Cl and Na acetate.

The response spectrum to α-amino compounds is not restricted. The imino acids, L-proline and hydroxy-L-proline, are both as effective as glycine, for example. Nonetheless, activity is clearly affected by certain structural modifications, as follows: (i) Increasing size of the molecule reduces activity. Among the α-amino acids this is most apparent in the instance of α-ϵ-diaminopimelic acid and L-α-aminoadipic acid. Maximal activity appears to occur in straight chain amino acids at C = 3 to 5. The dipeptides tested (Table VI) are all less active than their most active constituent amino acid, except possibly in the instance of L-lysyl-L-glutamic acid. Triglycine and tetraglycine exhibit progressively diminishing activities and polyglutamic acid and the two proteins studied were inactive.

TABLE VI

Activity ratios of peptides and proteins

Compound	Typical active constituent	AR	SD	N	t*
glycyl-glycine		0.66	0.12	25	
triglycine		0.50	0.09	15	
tetraglycine		0.40	0.10	12	—
	glycine	1.00	(arbitrary)		
glycyl-L-proline		0.36	0.10	10	0.001
	L-proline	1.00	0.17	14	
glycyl-DL-sarcosine		0.41	0.17	8	0.01
	DL-sarcosine	0.98	0.17	10	
glycyl-L-glutamic acid		0.41	0.11	10	0.001
	L-glutamic acid	1.37	0.29	12	
L-lysyl-L-glutamic acid		1.00	0.33	10	nsd
	L-glutamic acid	1.37	0.29	12	
polyglutamic acid (MW = 40,000–100,000) 25 mg./ml.		less than 0.1		5	
albumin, egg, 5 × cryst. 25 mg./ml.		less than 0.1		5	
haemoglobin, bovine, 2 × cryst. 25 mg./ml.		less than 0.1		5	

* Student's "t" test.

The inactivity of these last three substances might possibly be simply related to concentration. (ii) Only configurations approximating α-amino are effective. Consider in this regard the aminobutyric acids. DL-α-amino-n-butyric acid is the most active compound yet tested while γ-amino butyric acid is among the least active. α-amino isobutyric acid is also inactive, perhaps due to steric hindrance introduced by methyl substitution on the α-carbon. β-amino isobutyric acid, still quite active, would appear to be an exception to this proposition, except for the fact that this molecule can assume a configuration in which the carboxyl and β-amino groups are in the same steric relationship as in an α-amino compound. A similar argument is possible regarding β-alanine.

Certain other substituted compounds indicate the importance of the α-amino configuration to excitation. These include N-carbamyl and N-methyl substitutions, especially of glutamic acid, which reduce the AR from 1.37 to 0.20 and 0.24, respectively. Similarly, N-carbamyl glycine is quite inactive although N,N-dimethyl glycine appears to be an exception, as well as α-methyl DL-methionine. In the last instance the slight decrease in activity, if significant, might be due either to the α-methyl substituent or perhaps to D-methionine, whose activity has not been determined but which may well be less than L-methionine. Finally,

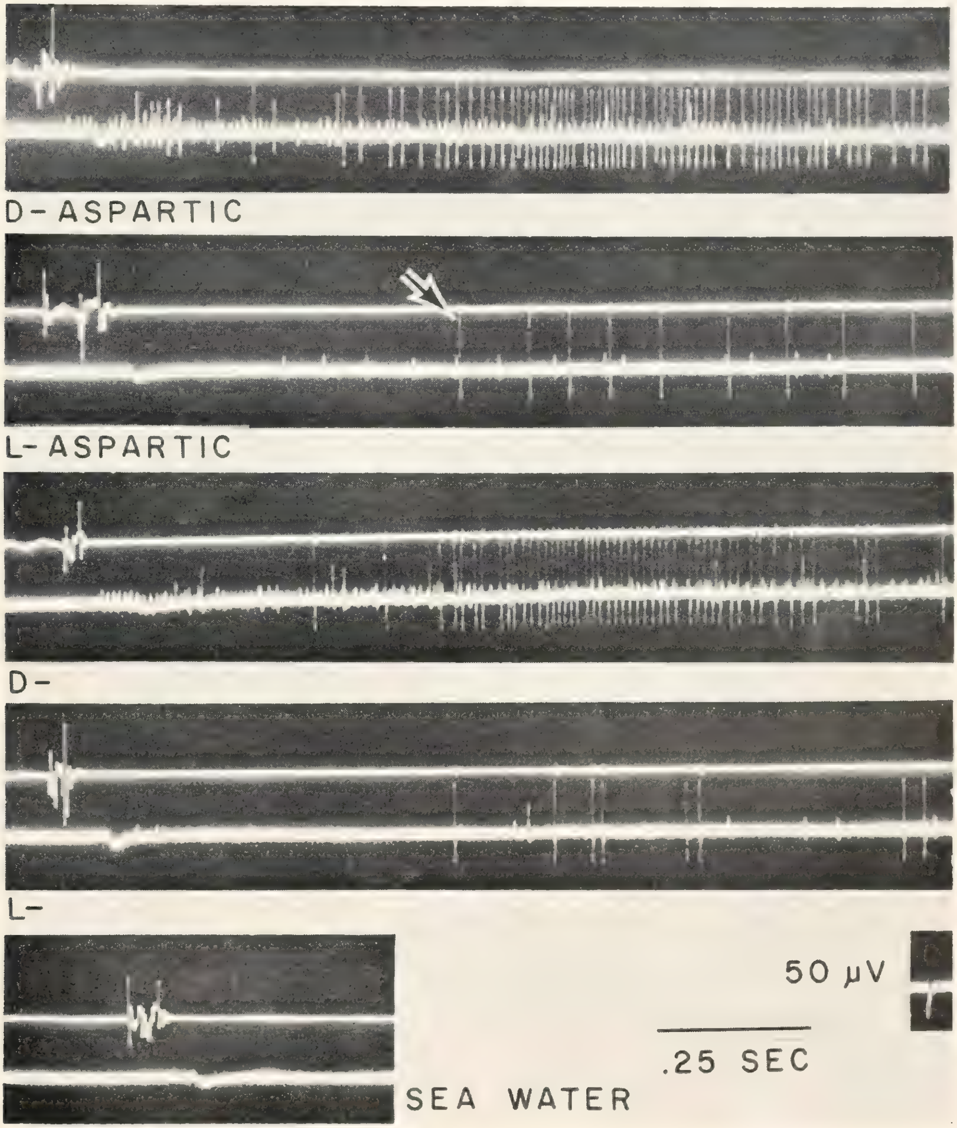


FIGURE 10. Demonstration of differential response of unit marked with arrow to D- and L-aspartic acid, 0.04 *M*. Records were taken sequentially with 5-second wash between each. *C. antennarius*.

even β- substitution may significantly impair activity, as in the instance of DL-β-methyl aspartic acid, although this effect is not realized in isoleucine which actually is as active as leucine and nor-leucine.

The data of Tables III, IV, and V demonstrate that members of several sterioisomer pairs do not have equal activities. D- is markedly more active than L-aspartic acid, while L- isomers of glutamic acid, leucine, iso-leucine and histidine are more active than their enantiomorphs. On the other hand, both isomers of alanine and valine are probably equally effective stimulants. At least in the instance of aspartic acid the differential sensitivity may be attributed to a single receptor cell as shown in Figure 10, where the unit indicated with an arrow is obviously differentially responsive to both isomers. When one isomer is markedly less active than the other, prior application of the less active isomer appears to have no inhibitory effect on response to the more active isomer. An experimental record shown in Figure 11 illustrates this point: Application of five drops of D-aspartic acid almost completely prevents response to L-aspartic acid, suggesting that both isomers activate the receptor at identical sites. The largely undiminished response

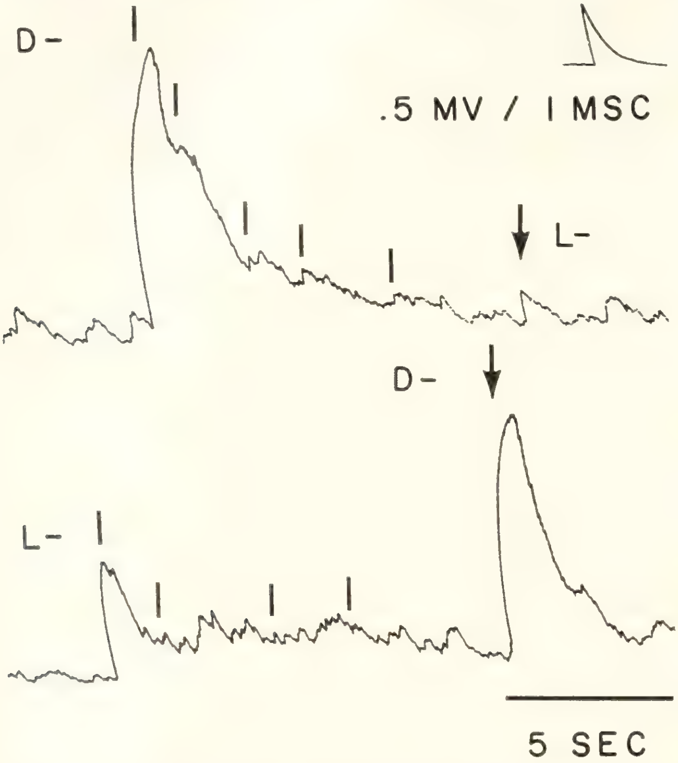


FIGURE 11. Demonstration that L-aspartic acid does not inhibit response to D-aspartic acid. Upper record, 5 drops 0.04 *M* D-aspartic acid, indicated by bars, followed without washing by 1 drop of 0.04 *M* L-aspartic acid, arrow. Lower record, 5 drops L-aspartic, at bars, followed without washing by 1 drop D-aspartic acid, at arrow. *C. antennarius.*

to D-aspartic acid after five drops of L-aspartic acid suggests further that the latter does not obstruct these sites.

Discussion

These experiments confirm the electrophysiological demonstration by Case and Gwilliam (1961) of the presence of amino acid-sensitive receptors on the dactyls of crabs, and depict the two species, *Carcinus* and *Cancer,* as markedly alike in chemosensory specificities. Even though the earlier work by Case and Gwilliam did not encompass as many substances as the present investigation and was only roughly quantitative, similarities between the two organisms are still obvious. The dactyl receptors of neither are responsive to such organic acids as α-keto glutaric or glutaric, or to certain sugars and alcohols. L-glutamic acid is an extremely effective stimulus to both and is in both considerably more excitatory than its sterioisomer. Similar responses to the isomers of aspartic acid occur in both although the differential sensitivity to leucine isomers reported for *Carcinus* appears to be reversed in *Cancer.* Other discrepancies are evident, but are difficult to interpret owing to the differences in methods of evaluating responses in the two reports. Particularly unfortunate was the use of one of the most effective compounds, L-glutamic acid, as the standard in the work on *Carcinus* since it undoubtedly rendered less precise the evaluation of weakly effective compounds.

Laverack (1963) was unable to confirm our observations on the dactyl chemoreceptors of *Carcinus* (Case and Gwilliam, 1961) and described in that organism yet another category of chemoreceptor, characterized by long response latencies on the order of 10–15 *seconds,* slow adaptation and sensitivity to only a few amines. Since the present work on another species demonstrates receptors quite similar to those which we found in *Carcinus,* Laverack's negative observations require little comment other than to note that his manner of preparing the dactyl differed considerably from ours. Dissection of the dactyl, the method employed by Laverack, is likely to be considerably more damaging to the receptor innervation than our method of subdivision of the nerve trunk more proximally.

Laverack's observation of long latency units is most interesting and may well represent an unusual class of chemoreceptors. We have so far failed to demonstrate such units although the response to trimethylamine, which he found an effective stimulant, was distinctly prolonged in our preparations. But even so the response is far too early to fall within the latency class he describes.

The magnitude of the externally recorded action potentials in the long latency units, 400 μV in one instance, is surprisingly large, as Laverack remarks, especially when one reflects on the physiological incongruity of providing such sluggish receptors with rapidly conducting axons. The largest unmistakable chemoreceptor spikes which we have observed are in the neighborhood of 100 μV in the most optimal external recording situations, and the majority are 50 μV or less. Large spikes are readily observed upon mechanical stimulation of a dactyl hair sensillum, and this, in conjunction with the long latency of Laverack's large units to chemical stimulation but *not* to mechanical (see our Fig. 5, for example), suggests the possibility that Laverack might have observed mechanoreceptors activated nonspecifically by chemical stimuli. While the responses figured by Laverack (his Fig. 6) do show an early burst of impulses, evidently associated with stimulus application and similar in spike size to the later chemical response, the data at the moment are insufficient to permit final determination of the problem.

The data of Table VI suggest that the dactyl receptors are not sensitive to peptides and proteins. While this seems quite plausible in the light of the sensitivity of the receptors to variation in structural details of the amino acids, it is also true that undoubtedly inappropriate proteins were utilized as test materials, owing to the problem of establishing purity. It must further be obvious that other receptor types may be present which do have sensitivity to peptides and proteins. Nevertheless, there appear to be no particular advantages for receptors mediating feeding responses to function as protein receptors as long as they are capable of detecting amino acids. Amino acids are considerably more soluble than proteins and hence are more likely to create a sensory trail to food sources. They lack the species specificity of proteins, which might introduce unwarranted specificity in the gustatory sense of the virtually omnivorous crabs.

While it is obviously unwise to argue from these observations that the arthropods in general do not possess protein receptors, there has been yet no proven instance of the electrophysiological demonstration of responsiveness to protein by an arthropod. Certainly the only other experiments known to us to be addressed to this question, those of Wallis (1961) on labellar chemoreceptor hairs of *Phormia*, in which haemoglobin and brain-heart infusion elicted neural activity, do not make a clear contribution to the problem. It is by no means obvious that these two substances, as applied, are amino acid-free, and they may not be free of other active contaminants, for example, the sugar and sodium chloride found in at least one commercial brain-heart infusion, which could be excitatory to the labellar receptors. There are, of course, compelling experiments in the behavioral literature which argue for the perception of host-specific, thermolabile materials by commensal organisms (Davenport, 1955). These may well be polypeptides or proteins.

Possible homology of neural junctions and sensory receptors (Grundfest, 1959) prompts inquiry into the similarities in the variety of amino acids to which they respond. Two classes of amino acid effects are evident at peripheral and central junctions: (1) excitatory, induced most strongly by dicarboxylic amino acids; and (2) inhibitory, principally due to the action of ω-amino acids. The dactyl chemoreceptors are responsive to many compounds from the first category and are not affected by ω-amino acids although certain compounds with junctional inhibitory effects are excitatory to them.

Robbins (1959) has reported excitation of crayfish muscle by L-glutamic acid at concentrations as low as 2×10^{-5} M with complete inactivity of D-glutamic acid. L-aspartic acid is somewhat less active than L-glutamic acid. The dactyl receptor threshold for L-glutamic acid is in the range of 10^{-5} M (Case and Gwilliam, 1961) and while D-glutamic acid is not without effect on the dactyl receptors, it is approximately three times less effective than the L-isomer. As in crayfish muscle, L-aspartic acid is considerably less effective than L-glutamic acid.

However, there is little correlation between inhibitory effectiveness of a compound on crayfish muscle and its excitatory effect on the dactyl receptors. Robbins (1959) lists in decreasing order of inhibitory effect on crayfish muscle: γ-aminobutyric acid > β-alanine > taurine > ϵ-aminocaproic acid. Taurine and β-alanine are highly effective dactyl receptor stimulants while the remaining members of the series are without effect. The divergence thus indicated is emphasized by the reactions of the two preparations to DL-α-aminobutyric acid. The most effective excitant known for the dactyl receptors, it has neither excitatory nor inhibitory influence on crayfish muscle.

The extensive investigation by Curtis, Phillis and Watkins (1961) of amino acid effects on toad spinal neurons similarly indicates better correlation of dactyl receptor response with excitatory rather than with inhibitory compounds. Yet the correlations are by no means adequate enough to argue for more than the most general similarity in excitatory mechanisms between the two preparations. The great sensitivity of dactyl chemoreceptors to α-amino monocarboxylic acids, as well as to dicarboxylic acids, constitutes a major difference in the two preparations. Further divergence is seen in the insensitivity of toad spinal neurons to N-substitution in active compounds, this generally serving to impair activity in the dactyl preparation. Another dissimilarity lies in the generally greater excitatory effects of D- isomers on the toad neuron. The dactyl receptor may be differentially sensitive to either optical isomer or to neither.

As far as compounds inhibitory to toad spinal neurons are concerned, the correlation is as negative as between dactyl receptors and crayfish muscle. Taurine, β-alanine and γ-aminobutyric acid are all strongly inhibitory to the spinal neuron, while only γ-aminobutyric acid lacks an excitatory effect on dactyl receptors.

Finally, the possibility has been tested that junctional inhibitors, while lacking excitatory effects on the dactyl chemoreceptors, might reduce dactyl responses to effective stimulants (Case and Miller, unpublished observations). Pretreatment of dactyl receptors with γ-aminobutyric acid had no observable effect on responses to effective amino acids. The dactyl receptors are thus categorized as primarily responsive to α-amino acids with specificity sufficiently broad to include many junctional excitants but not the inhibitory ω-amino acids.

SUMMARY

Chemoreceptors present on the distal limb segments of *Cancer antennarius* and *C. productus* have been examined by recording from axons dissected from the limb nerve. Receptor latencies are on the order of 35 msec., depending upon stimulant and concentration, and adaptation is rapid. Response intensity is linearly related to the logarithm of concentration. Optical isomers of certain compounds are discriminated and in one instance response is shown to be pH-dependent. Most effective stimulants are α-amino acids and related compounds. Among the most effective compounds are DL-α-amino-N-butyric acid, taurine, L-glutamic acid, and serine, in descending order of activities. Peptides are uniformly less active than their constituent amino acids, and two proteins were found to be without activity.

LITERATURE CITED

BARBER, S. B., 1961. Chemoreception and thermoreception. Pp. 109–131 in The Physiology of Crustacea, Vol. II (Ed. T. H. Waterman), Academic Press, New York.

CASE, J., 1964. Adequate stimulus of certain *Cancer* dactyl chemoreceptors. *Amer. Zool.,* **4**: 37.

CASE, J., AND G. F. GWILLIAM, 1961. Amino acid sensitivity of the dactyl chemoreceptors of *Carcinides maenas*. *Biol. Bull.,* **121**: 449–455.

Case, J., and G. F. Gwilliam, 1963. Amino acid detection by marine invertebrates. *Proc. XVI Intern. Cong. Zool.*, p. 47.

Case, J., G. F. Gwilliam and F. Hanson, 1960. Dactyl chemoreceptors of brachyurans. *Biol. Bull.*, **119**: 308.

Curtis, D. R., J. W. Phillis and J. C. Watkins, 1961. Actions of amino-acids on the isolated hemisected spinal cord of the toad. *Brit. J. Pharmacol.*, **16**: 262–283.

Davenport, D., 1955. Specificity and behavior in symbiosis. *Quart. Rev. Biol.*, **30**: 29–46.

Grundfest, H., 1959. Synaptic and ephaptic transmission. Pp. 147–198 in Handbook of Physiology, Vol. 1, Sect. 1 (Ed. J. Field), American Physiological Society.

Hodgson, E. S., 1958. Electrophysiological studies of arthropod chemoreception. III. Chemoreceptors of terrestrial and fresh water arthropods. *Biol. Bull.*, **115**: 114–125.

Laverack, M. S., 1963. Aspects of chemoreception in crustacea. *Comp. Biochem. Physiol.*, **8**: 141–151.

Luther, W., 1930. Versuche über die Chemorezeption der Brachuren. *Zeitschr. vergl. Physiol.*, **12**: 177–205.

Robbins, J., 1959. The excitation and inhibition of crustacean muscle by amino acids. *J. Physiol.*, **148**: 39–50.

Wallis, D. I., 1961. Responses of the labellar hairs of the blowfly, *Phormia regina* Meigen to protein. *Nature*, **191**: 917–918.

TRENDS IN WATER AND SALT REGULATION AMONG AQUATIC AND AMPHIBIOUS CRABS

WARREN J. GROSS

Division of Life Sciences, University of California, Riverside, California

The evolutionary invasion of land from an aquatic environment has required the development of physiological mechanisms to maintain a high degree of homeostasis in the extreme stresses of the aerial environment. Yet successful land animals can be examined only in their present perfected states of terrestrialness, the steps taken to reach this degree of perfection being obscured from direct observation. On the other hand, in a few taxonomic groups members representing different degrees of terrestrialness are common and a study of an array of such transitional forms may reveal possible physiological steps by which the attainment of the land habit can be made. The decapod Crustacea include groups such as the brachyuran and anomuran crabs, which present a full spectrum of intermediaries between aquatic and terrestrial forms. Yet relatively little attention has been paid to the physiology of these transitional forms. The subject of terrestrialness in crustaceans is reviewed by Edney (1960). Pearse (1934) pointed out the tendency for crabs showing degrees of terrestrialness to maintain the osmotic concentration of their blood below that of the sea. Jones (1941) showed a correlation between terrestrialness among crabs and their ability to maintain their blood below the osmotic concentration of a hypersaline medium (hyporegulation). The adaptive significance of hypoosmotic regulation among terrestrial and semi-terrestrial crabs has been questioned (Gross, 1955), but in turn this correlation was explained in part as a reflection of the evolutionary history of the species as well as, or even apart from, their present requirements (Gross, 1961). The role of behavior in water regulation among terrestrial crabs has been demonstrated (Gross and Holland, 1960; Gross, 1964). Ionic and water regulation in the semi-terrestrial crab, *Pachygrapsus crassipes,* has been examined (Jones, 1941; Prosser *et al.,* 1955; Gross, 1957, 1958, 1959); members of the semi-terrestrial genus, *Uca,* have been studied with respect to osmotic regulation in *Uca crenulata* (Jones, 1941), osmotic and ionic regulation in *Uca pugnax* and *U. pugilator* by Green *et al.* (1959). Chloride regulation has been studied in the semi-terrestrial *Ocypode quadrata* (Flemister and Flemister, 1951; Flemister, 1958) and also in the semi-terrestrial *Goniopsis cruentatus,* as well as the extreme land crab, *Gecarcinus lateralis* (Flemister, 1958). Cation and water balance in *Gecarcinus* exposed to various terrestrial environments has been investigated by Gross (1963a). DeLeersnyder and Hoestlandt (1963) studied osmotic and ionic regulation in the land crab, *Cardisoma armatum.*

Evidence for an osmo-regulatory function of the crab antennary gland is known in only a few cases (Lockwood, 1962). For example, Green *et al.* (1959) report that *Uca pugilator* and *U. pugnax* when immersed in 175% sea water maintain their urine hypertonic to the blood. However, there is considerable evidence that the primary function of the crab antennary gland is that of ionic regulation (Prosser and Brown, 1961). Of particular interest is the relationship between the concentration of Mg and Na in the urine of certain amphibious crabs when they are immersed in media of different salinities. Thus, it was demonstrated in *Pachygrapsus* by Prosser *et al.* (1955) that as the medium becomes more concentrated, the urine Mg increases in concentration, but the urine Na decreases in concentration. Such a phenomenon was confirmed in *Pachygrapsus* by Gross (1959), in two species of *Uca* by Green *et al.* (1959), and also a similar phenomenon was found in the mud crab, *Hemigrapsus oregonensis,* by Gross (1961) who interpreted the ability to concentrate Mg in the urine as an adaptation to hypersaline water which in turn would be correlated with the ability to hyporegulate osmotically.

The present investigation further explores the correlation between hypoosmotic regulation, Mg regulation and attainment of the terrestrial mode of life. Thus, seven species of crabs which ranged from the completely aquatic to the most terrestrial were studied.

Materials and Methods

The following species of crabs, which are the principal subjects of this study, are ordered according to their estimated degree of terrestrialness. The completely aquatic *Cancer antennarius* Stimpson was collected at Laguna, California; *Hemigrapsus oregonensis* (Dana) was collected at Newport, California; *Pachygrapsus crassipes* Randall was collected at Newport and Laguna, California; *Grapsus grapsus tenuicrustatus* (Herbst) and *Ocypode ceratophthalma* (Pallas) were both captured at Eniwetok Atoll, Marshall Islands, and studied in the Eniwetok Marine Biological Laboratory. *Uca crenulata* (Lockington) was collected at Newport, California, and finally *Gecarcinus lateralis* (Fréminville), probably the most terrestrial of all crabs, was collected in Bermuda and flown to Riverside where it was maintained and studied in the laboratory.

The relative degree of terrestrialness in some of the above species may be debated, but consideration has been given not only to the tidal level of their habitats but also to their diurnal activities. That is, a diurnal animal on land would be considered more exposed to the rigors of terrestrialness than a nocturnal animal. A brief description of the crab habitats is given in Table I.

All crabs used in these experiments were mature and between molts. *Gecarcinus* had been maintained in the laboratory for several weeks in pens before use. Specimens of *Cancer* exposed to high salinities (see below) were maintained in a running sea water system for a few weeks. All other crabs used in this investigation were freshly collected, and maintained for only brief periods (less than two weeks) in the laboratory in 100% natural sea water.

To demonstrate the response to an osmotic stress, all animals except *Gecarcinus* and *Cancer* were transferred from normal sea water and immersed in different salinities. *Gecarcinus,* which normally does not enter water (Rathbun, 1918; Bliss, 1962), was transferred to the aqueous media of different salinities from a terrestrial situation where it had access to fresh water, sea water and food. *Cancer* was maintained for several weeks in a running sea water system which increased in concentration to 115% sea water by evaporation. Specimens of *Cancer* studied in 100% sea water were freshly collected. With the exception of the land crab, *Gecarcinus,* 100% sea water was considered to be close to the natural medium for all species examined, and the responses to 100% sea water indicated below, unless otherwise stated, are for exposure to this salinity for an indefinite period. Exposure to stress media (other than normal sea water) was for 24 hours for all species except *Uca* and *Gecarcinus*. *Uca* was immersed in the stress media for 48 hours. This period of exposure was arbitrarily selected early in the investigation, but changed to the 24-hour period for the other species in anticipation of a high attrition rate for the weak regulators during the longer period of immersion. *Gecarcinus* was exposed to 100% sea water for the prescribed 24-hour period. However, in 50% and 150% sea water this crab was immersed only 8 to 10 hours. *Gecarcinus* did not survive longer periods of immersion in these stress media.

TABLE I

Species	Habitat
Cancer antennarius	Subtidal levels, always submerged in water or burrowed in wet sand of rocky-sandy beaches. Rarely exposed to anything but normal sea water.
Hemigrapsus oregonensis	Burrows in mud of back bay areas. Usually submerged, but occasionally exposed at low tide. Found in brackish and hypersaline water.
Pachygrapsus crassipes	Semi-terrestrial. Found on rock shores and in back bays. Spends considerable time out of the water both day and night but close to water's edge. Predominantly nocturnal in its terrestrial behavior. Found in hypersaline water and possibly brackish water.
*Grapsus grapsus**	Semi-terrestrial. Found at about the same tidal level as *Pachygrapsus*, but is more diurnal in its terrestrialness than *Pachygrapsus*.
*Ocypode ceratophthalma**	Burrows high on sandy beaches. Occasionally active on the beach during the daytime, but usually at night.
Uca crenulata	Burrows high in muddy sand of back bays. Exposed commonly in the daytime. Not commonly seen at night. Found in hypersaline water and possibly brackish water.
Gecarcinus lateralis	Probably the most terrestrial of all crabs. Rarely enters water and can live indefinitely with damp sand as its only source of water.

*No information is available concerning the extremes of salinity experienced by these crabs; however, they inhabit areas of torrential rains and undoubtedly endure rapid changes in salinity.

The volume of medium used in these experiments was sufficient to cover the animal in all cases except *Gecarcinus*, which had to be permitted to rise out of the water to prevent drowning.

The temperatures used for the test media were close to those commonly found in the natural habitats of the respective species; they were as follows: *Cancer*, 20° C.; *Hemigrapsus*, 15° C.; *Pachygrapsus*, 15° C.; *Grapsus*, 26° C.; *Ocypode*, 26° C.; *Uca*, 15° C., and *Gecarcinus*, 23° to 25° C.

After a given period of immersion, the blood and urine of the crabs were sampled and analyzed for Na, K, Ca, Mg and osmotic concentration. Blood was extracted from all species by puncturing the arthrodial membranes at the bases of the appendages with a glass pipette. Urine was removed from the nephropores by means of a fine glass cannula. However, in the cases of *Gecarcinus* and *Uca* the opercula of the nephropores were removed about two days before an experiment (Gross, 1963a). This permitted the insertion of the cannula without forcing open the operculum, an operation which usually resulted in a loss of blood. Urine could thus be collected without a mixture of blood. Inasmuch as urine is clear and blood turbid, contamination could always be detected and doubtful samples discarded.

Na and K were analyzed by flame photometry; Ca and Mg by ethylene diamine tetra-acetic acid (EDTA) titration as previously described (Gross, 1959). Osmotic concentrations were determined by means of a Mechrolab vapor pressure osmometer. For blood this required removal of the clot. However, there is good evidence that there is an insignificant osmotic difference between whole blood and serum of crabs (Prosser *et al.*, 1955; Gross, 1963b).

For both blood and urine in the concentration range of normal sea water, Na could be determined to an accuracy of about 2% error, K less than 10% error, Ca and Mg less than 6% error, osmotic concentration about 1% error. Because of the small size of *Uca*, blood samples had to be pooled from two to four animals for Mg and Ca determinations. Cation analyses of samples of blood and urine collected at Eniwetok from *Grapsus* and *Ocypode* were unreliable and will not be presented. Normal sea water was considered to contain 3.43% salt.

RESULTS

Table II presents values for osmotic concentrations of body fluids for six species of crabs immersed in different salinities. The blood concentrations are compared in Figure 1. *Cancer antennarius* is not included in Table II because it is a non-regulator (Jones, 1941) and cannot stand the osmotic stresses imposed on the other species. Its blood, therefore, is considered to be essentially isotonic with the medium in all viable concentrations. Osmotic determinations were not made on the body fluids of *Gecarcinus*. However, for the blood, these can be estimated from the total cation concentrations (m*M*./l.) which, if expressed as percentage of those in normal sea water, approximate the osmotic concentration (Gross, 1961). Thus, the means for *Gecarcinus* in Figure 1 and Table II were calculated from the values for total cations (m*M*./l.) in Table VII.

First it should be emphasized that comparison of the response of these animals to osmotic stress is meaningful only for the specific set of conditions used in these studies, for as shown by Gross (1963b), *Hemigrapsus* hyporegulates more strongly when exposed to high salinities for prolonged periods than it does when immersed suddenly into hypersaline water. It may be that other species acclimate in a similar way or even in a reverse manner. Nevertheless, the responses of *Pachygrapsus* illustrated in Figure 1 compare favorably with those previously reported (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957). Likewise the behavior of *Uca* (Fig. 1) was about the same as demonstrated by Jones (1941). The value for blood osmotic concentration in *Grapsus* immersed in normal sea water was almost the same as that reported by Pearse (1934) for the West Indies form. On the other hand, Figure 1 shows that *Hemigrapsus* can hyporegulate in concentrated sea water. This is contrary to the findings of Jones (1941). However, this conflict of findings has been explained by Gross (1963b).

Thus, Figure 1 and Table II demonstrate that all crabs examined, showing some degree of terrestrialness, are hypoosmotic regulators as well as hyperosmotic regulators. This confirms and expands a phenomenon first reported by Jones (1941). Also, it will be noted that there is some correlation between degree of hypoosmotic regulation and the degree of terrestrialness. This correlation as illustrated in Figure 1 is far from perfect, for *Gecarcinus,* which undoubtedly is the most terrestrial, was only the third strongest hyporegulator. Also, it will be recalled that this crab was incompletely immersed in 50% and 150% sea water for only 8 to 10 hours rather than 24 hours. It therefore seems that the ability to osmoregulate in this land crab is not as powerful as indicated in Figure 1. Also, *Uca* according to Figure 1 does not maintain its blood as low in concentration in hypersalinities as does *Ocypode;* still, *Uca* has been considered to be somewhat more exposed to terrestrial stresses than *Ocypode* because of its diurnal activity. On the other hand it can be said that the most terrestrial crabs, with the possible exception of *Gecarcinus,* were stronger hyporegulators for these

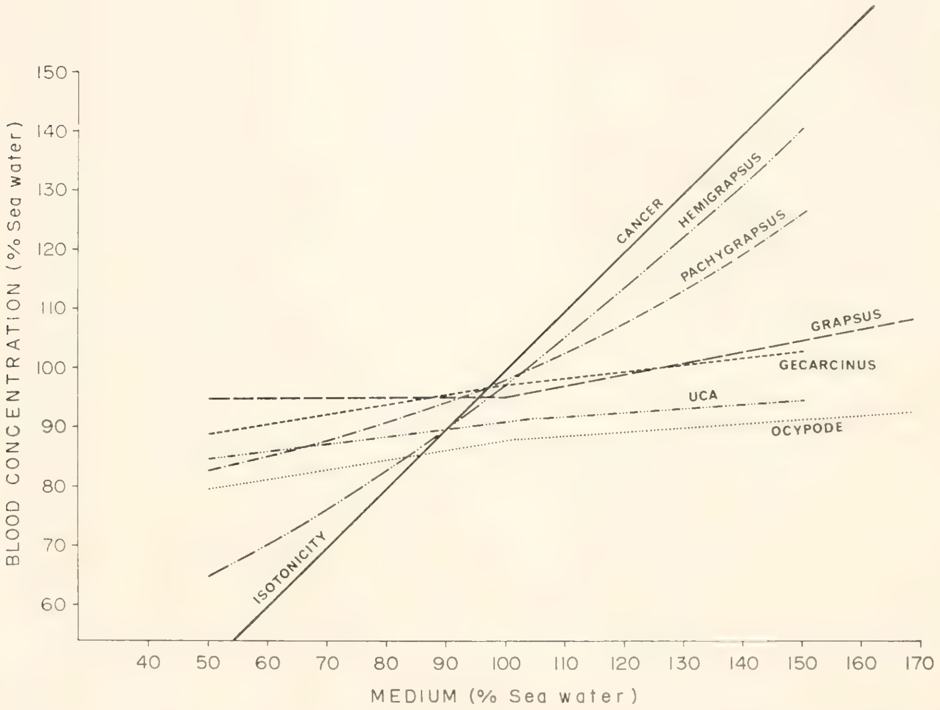


FIGURE 1. Comparative osmoregulatory ability of crabs showing different degrees of terrestrialness. Habitats of crabs are described in text and in Table I. Curves are estimated from mean values in Table II.

sets of conditions than the most aquatic crabs. It is also apparent that *Grapsus, Uca,* and *Ocypode* were powerful osmotic regulators in all test salinities. Also, as observed by Pearse (1934), in another group of animals, there is a tendency for amphibious crabs to maintain their blood hypotonic to a medium of normal sea water (Table II, Fig. 1). Jones (1941) indicated that among amphibious crabs the strongest hyporegulators were also the strongest hyperregulators. If the magnitude of the osmotic gradient sustained between blood and external medium is considered to be the degree of regulation, then *Ocypode,* which would be the strongest hyporegulator, would be next to the weakest hyperosmotic regulator; *Grapsus* would be the fourth strongest hyporegulator and the best hyperregulator.

TABLE II

*Effects of stress on osmotic concentration in body fluids of immersed crabs**

		Medium % sea water											
		50			100			150			168		
		Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.
Hemigrapsus	Blood (% SW)	65.0	5	3.00	97.3	10	1.92	141	10	4.10			
	U/B	1.09	5	(1.00 1.16)	1.02	5	(1.01–1.04)	1.01	4	(0.91–1.06)			
Pachygrapsus	Blood (% SW)	82.6	6	6.81	98.3	5	1.66	127	6	5.88			
	U/B	1.00	5	(0.98-1.03)	0.97	5	(0.94–0.99)	0.96	5	(0.94–0.97)			
Grapsus	Blood (% SW)	94.9	5	5.62	93.5	11	4.16				109	9	13.9
	U/B	0.98	4	(0.96–1.01)	1.01	11	(0.98–1.05)				0.97	8	(0.92–1.02)
Ocypode	Blood (% SW)	79.9	6	2.24	87.6	6	2.61				93.2	6	6.51
	U/B	1.00	5	(0.99–1.02)	0.99	5	(0.97–1.01)				1.00	5	(0.98–1.01)
*Uca***	Blood (% SW)	84.6	7	4.00	90.7	6	3.82	94.7	12	2.54			
	U/B	0.98	4	(0.97–1.00)	–	–	–	0.96	4	(0.87–1.03)			
Gecarcinus§	Blood (% SW)	87.8	12	5.34	95.0	11	5.79	103	15	10.6			

U/B = Urine concentration/blood concentration.

**Uca* was completely immersed for 48 hours; *Gecarcinus* was partially immersed for 24 hours in 100% sea water and 8–10 hours in 50% and 150% sea water; all other crabs were exposed to stress media for 24 hours.

**U/B values for *Uca* were determined on crabs immersed for 24 hours.

Values in parentheses = range.

§Estimated from total cations.

Even if deviation from normal blood concentrations (those when immersed in 100% sea water) were considered the criterion for degree of regulation, *Grapsus* would be the strongest hyperregulator but not the strongest hyporegulator. The significance of difference in osmoregulation for the respective species is presented in Table III A.

Considering the ratio, urine osmotic concentration/blood osmotic concentration (U/B values) (Table II), it is apparent that the curves for urine would be essentially the same as those for blood in *Hemigrapsus, Pachygrapsus, Grapsus, Uca* and *Ocypode* illustrated in Figure 1. That is, the U/B values were close to unity in all five species for all conditions studied. An examination of the cation U/B values for all the treatments in the case of *Gecarcinus* (Table VII) reveals that the osmotic concentration of the urine could not differ greatly from the blood when this crab is immersed. Osmotic U/B values for this crab in a terrestrial situation have been shown to be essentially unity (Gross, 1963a).

It is therefore apparent that the antennary glands of the above crabs do not serve a significant osmoregulatory function. This is in agreement with previous findings on *Pachygrapsus* (Jones, 1941; Prosser *et al.*, 1955). Contrary to findings in the present investigation on *Uca crenulata,* Green *et al.* (1959) reported osmotic U/B values significantly greater than one in *Uca pugnax* and *Uca pugilator,* when they were immersed in concentrated sea water. Such values, however, were sufficiently close to unity to be of questionable significance physiologically. Flemister (1958) reports that *Ocypode quadrata* could not regulate its blood chloride for 24 hours in about 130% sea water. However, as shown in Table II and Figure 1, *Ocypode ceratophthalma* showed almost perfect osmotic regulation in 168% sea water for 24 hours. Also, exploratory experiments in this laboratory on *Ocypode quadrata* from Bermuda showed that four specimens of this crab immersed in 150% sea water for 72 hours held their blood Na at least 20% below the Na concentration in the medium. Flemister (1958) reported sig-

TABLE III

Probability values

A. Blood osmotic concentrations	Concentration of medium (% sea water)			
	50	100	150	168
Hemigrapsus v. *Pachygrapsus*	<0.001	not sig.	<0.001	
Pachygrapsus v. *Grapsus*	<0.01	<0.01	<0.01*	
Grapsus v. *Uca*	<0.01	not sig.	not sig.*	
Uca v. *Ocypode*	<0.05	not sig.	—	
Grapsus v. *Ocypode*	<0.001	<0.01	—	<0.02
Gecarcinus v. *Grapsus*	<0.05	not sig.	—	
Gecarcinus v. *Uca*	not sig.	not sig.	<0.01	
Gecarcinus v. *Pachygrapsus*	not sig.	not sig.	<0.001	
Gecarcinus v. *Ocypode*	<0.001	<0.01	<0.02*	

*Crabs in 150% sea water compared with *Grapsus* and *Ocypode* in 168% sea water.

B. Na U/B values	50% SW v. 100% SW	100% SW v. 150% SW
Hemigrapsus	<0.001	<0.001
*Pachygrapsus**	<0.001	<0.01
Uca	**	**
Gecarcinus	not sig.	not sig.
	100% SW v. 115% SW	
Cancer	<0.05	

*Calculated from Gross (1959).

**U/B values for Na were not determined on individual specimens of *Uca*.

nificantly higher values for urine chloride than for blood chloride in *Ocypode,* thus suggesting an osmoregulatory function of the antennary glands. However, considering the most extreme U/B ratio for *Ocypode ceratophthalma,* 1.02 (Table II), there is no indication of an osmoregulatory role of the antennary gland in this species.

Tables IV, V, VI and VII demonstrate the effect of osmotic stress on the cation concentrations of blood and urine in *Cancer, Hemigrapsus, Uca* and *Gecarcinus,* respectively.

With regard to the regulation of K, there were no apparent trends with increasing terrestrialness, either with respect to blood levels or urine, or U/B values. As indicated in Table VII, above, only *Gecarcinus* maintained its blood K below that of the external medium when immersed in 100% sea water (8.77 meq./l. to 9.8 meq./l.). This also was demonstrated by *Pachygrapsus* (7.43 meq./l. to 9.8 meq./l.) (Gross, 1959). The mean U/B values for K in *Gecarcinus* were greater than unity for all treatments, but significantly so only when immersed in 50% and 100% sea water ($P < 0.01$). The mean U/B values for K in *Uca* were greater than one for all treatments ($P < 0.001$). This suggests a K-regulating role of the antennary gland for these two crabs. *Hemigrapsus,* on the other hand, demonstrated mean U/B values for K which are significantly less than unity in 100% sea water ($P < 0.05$) and in 150% sea water ($P < 0.001$), suggesting conservation of this ion by the antennary gland. However, attention

TABLE IV

*Effects of stress on ionic concentrations in blood and urine of immersed Cancer antennarius**

		A Normal (100% SW)			B 115% SW			C 100% SW 1.5 × Mg			D 100% SW Mg FREE		
		Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.
Na meq./l.	U	480	4	17.0	451	5	36.5	339	4	89.4	473	5	63.2
	B	492	5	11.6	509	5	9.00	456	4	13.1	481	5	34.6
	U/B	0.98	4	0.04	0.88	5	0.07	0.82	3	0.19	0.99	5	0.19
	Medium	464			534			427			542		
K meq./l.	U	9.75	4	0.39	11.5	5	0.49	10.4	4	1.39	16.9	5	9.38
	B	10.7	5	0.69	12.6	5	0.40	11.5	4	0.62	13.0	5	1.41
	U/B	0.93	4	0.02	0.91	5	0.06	0.92	3	0.15	1.33	5	0.81
	Medium	9.81			11.3			9.8			9.8		
Ca meq./l.	U	27.2	3	5.28	24.8	5	0.95	26.6	4	1.17	27.5	5	5.08
	B	27.3	5	3.04	26.0	5	1.79	24.7	4	0.32	27.6	5	6.24
	U/B	1.03	3	0.37	0.96	5	0.08	1.08	3	0.15	1.01	5	0.14
	Medium	20.0			23.0			20.0			20.0		
Mg meq./l.	U	102	3	18.9	163	5	44.6	328	4	131	158	5	34.6
	B	61.1	5	6.81	59.5	5	4.20	86.3	4	2.20	58.6	5	5.45
	U/B	1.74	3	0.24	2.82	5	1.01	3.29	3	1.04	2.70	5	0.15
	Medium	104			120			156			0		

*Periods of immersion were: A, indefinite; B, at least two weeks; C, 18 hours; D, 4 hours.

should be called to the U/B value for K (1.69) when *Hemigrapsus* was immersed in 50% sea water (Table V). It would seem that if these ratios were physiologically adaptive, the higher values would be found in higher salinities and *vice versa*. *Pachygrapsus* showed the same pattern as *Hemigrapsus* in this regard, that is, U/B values less than one were demonstrated in 100% and 150% sea water and values greater than one in 50% sea water (Gross, 1959). The physiological significance of these ratios cannot be resolved until rates of K flux are known, as well as values for urine flow for the same conditions.

Considering the blood Ca concentrations when the animals were immersed in 100% sea water, there was an apparent trend with respect to terrestrialness. Thus, for the aquatic *Cancer,* the mean blood Ca was 27.3 meq./l. (Table IV); for *Hemigrapsus,* 29.5 meq./l. (Table V); for *Pachygrapsus,* 29.6 meq./l. (Gross, 1959); for *Uca,* 39.3 meq./l. (Table VI) and finally for the terrestrial *Gecarcinus* 44.2 meq./l. (Table VII). Although the mean Ca concentrations in the blood of *Cancer, Hemigrapsus* and *Pachygrapsus* were not significantly different from each other, the blood Ca of *Uca* was significantly greater than that of *Pachygrapsus* ($P < 0.001$) and significantly less than that of *Gecarcinus* ($P < 0.05$). If Ca regulation in the stressed media of 50% and 150% sea water is considered, *Cancer* must be omitted from comparison because of its inability to tolerate such extremes of salinity. However, the apparent correlation holds true with this omission in 50% sea water, but in 150% sea water *Pachygrapsus* showed the mean blood Ca concentration, 36.4 meq./l. (Gross, 1959) compared to 36.1 meq./l. for *Uca*

TABLE V

Effects of stress on ionic concentrations in blood and urine of Hemigrapsus oregonensis immersed for 24 hours

		50% SW			100% SW			150% SW		
		Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.
Na meq./l.	U	376	11	47.5	410	15	67.1	420	10	75.0
	B	331	11	48.5	488	15	19.9	685	11	13.8
	U/B	1.14	11	0.11	0.84	12	0.12	0.61	10	0.10
	Medium	232			464			696		
K meq/l.	U	9.75	11	6.89	9.37	15	2.06	12.03	10	1.78
	B	6.63	11	0.79	10.79	15	1.44	15.84	11	3.98
	U/B	1.69	11	0.87	0.85	12	0.22	0.77	10	0.14
	Medium	4.9			9.8			14.7		
Ca meq./l.	U	37.3	10	5.46	34.0	15	10.44	49.9	11	10.67
	B	30.8	10	7.46	29.5	12	4.24	33.6	11	4.51
	U/B	1.31	9	0.25	1.14	9	0.42	1.50	11	0.35
	Medium	10.0			20.0			30.0		
Mg meq./l.	U	78.1	10	21.1	261	15	109	480	10	165
	B	34.3	10	5.06	47.0	12	10.25	61.1	11	9.12
	U/B	2.43	9	1.50	4.96	9	2.24	7.76	9	3.07
	Medium	52			104			156		

Table VI), values which are not significantly different. *Gecarcinus* then also had the highest mean blood Ca in 150% sea water (44.2 meq./l.). It should be emphasized, however, that the most valid comparison can be made for crabs immersed in 100% sea water, a treatment to which *Gecarcinus* was subjected the longest period (24 hours); yet even here, it must be recalled that *Gecarcinus* was not completely immersed for any treatment. It is of interest that in all species examined blood Ca remained relatively constant even though the other ions were forced away from normal by the osmotic stress. The same type of constancy for blood Ca has been described in *Cardisoma armatum*, but the low Ca concentration in the blood of this land crab does not fit the above correlation with respect to terrestrialness (DeLeersnyder and Hoestlandt, 1963).

Table VI

Effects of stress on ionic concentrations in blood and urine of Uca crenulata immersed for 48 hours

		50% SW			100% SW			150% SW		
		Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.
Na meq./l.	U	280	11	51.5	271	15	59.4	301	12	88.2
	B	374	11	28.9	420	10	19.6	492	10	10.5
	U/B*	0.75			0.65			0.61		
	Medium	232			464			696		
K meq./l.	U	15.1	11	3.05	20.1	15	6.28	19.8	12	4.87
	B	9.51	11	1.29	11.4	10	1.70	12.2	10	1.99
	U/B*	1.59			1.87			1.62		
	Medium	4.9			9.8			14.7		
Ca meq./l.	U	24.7	12	10.5	27.0	12	3.95	25.9	11	4.62
	B	37.0	10	8.20	39.3	19	6.95	36.1	10	4.80
	U/B*	0.67			0.64			0.72		
	Medium	10.0			20.0			30.0		
Mg meq./l.	U	134	12	48.1	347	12	93.2	563	11	113
	B	23.8	10	6.90	61.3	19	17.3	85.0	10	33.6
	U/B*	4.73			5.32			6.62		
	Medium	52.0			104			156		

*Mean urine concentration/mean blood concentration, not mean U/B ratio.

There were interesting differences in the response of the antennary glands of the different species with respect to Ca. For example, the mean Ca U/B values for *Uca* were significantly less than unity for all media studied ($P < 0.001$), but in *Hemigrapsus*, they were greater than unity for all conditions examined although only in 50% and 150% sea water are the ratios significantly different from unity ($P < 0.01$). In *Pachygrapsus*, as shown by Gross (1959), the mean Ca U/B value, when the crab was immersed in 50% sea water, was not significantly different from one, but was greater than unity for the treatment in 100% and 150% sea water ($P < 0.01$). Finally, in the case of *Gecarcinus* the mean Ca U/B value was less than unity for the treatment in 100% sea water, but not significantly different from unity for the other treatments. Again, however, the physiological significance of these U/B values awaits further studies on the fluxes of this ion and on the rates of urine flow.

The regulation of Na and Mg is of particular interest in this study. As expected, the blood Na increased with increasing salinity of the external medium in all species studied, reflecting the blood osmotic concentration (Table II); yet the urine Na did not increase as much proportionately as the blood Na in the cases of *Cancer, Hemigrapsus,* and *Uca* (Tables IV, V and VI). Prosser *et al.* (1955) reported that the urine Na of *Pachygrapsus* dramatically decreased when the external medium was increased in concentration and Gross (1959) showed a similar but less dramatic effect in *Pachygrapsus*. In the three above species, however, there were no significant decreases in urine Na with increasing salinities. Although *Uca* showed a lower mean urine Na concentration in 100% sea water than in 50% sea water, and *Cancer* showed a lower mean urine Na concentration in 115% sea water than in 100% sea water, such differences were not significant. However, considering the mean U/B ratios for Na, *Cancer, Hemigrapsus,* and *Uca* demonstrated significant decreases (Table III B) as the salinity of the medium was increased; such was also the case for *Pachygrapsus* (Prosser *et al.*, 1955; Gross, 1959). Yet, this was not so for *Gecarcinus,* which revealed mean U/B values for Na which were about equal and significantly above unity for all

TABLE VII

*Effects of stress on ionic concentrations in blood and urine of Gecarcinus lateralis**

		50% SW			100% SW			150% SW		
		Mean	No.	S.D.	Mean	No.	S.D.	Mean	No.	S.D.
Na meq./l.	U	453	12	39.9	499	13	20.8	520	10	29.3
	B	435	14	27.7	468	13	27.9	507	15	52.1
	U/B	1.07	11	0.07	1.08	10	0.04	1.08	10	0.03
	Medium	232			464			696		
K meq./l.	U	9.92	11	2.32	12.1	13	3.82	10.7	10	4.65
	B	8.40	14	1.32	8.77	13	0.71	9.42	15	1.43
	U/B	1.29	11	0.30	1.48	10	0.42	1.18	10	0.44
	Medium	4.9			9.8			14.7		
Ca meq./l.	U	40.0	11	21.3	33.9	12	15.3	37.9	12	14.2
	B	41.4	14	8.48	44.2	11	5.41	44.2	15	7.52
	U/B	1.04	11	0.53	0.65	9	0.26	0.71	5	0.25
	Medium	10.0			20.0			30.0		
Mg meq./l.	U	48.8	11	27.3	41.5	12	22.5	54.2	12	25.2
	B	19.3	12	9.70	27.8	11	8.30	29.8	15	9.70
	U/B	2.60	11	1.29	1.74	9	0.59	2.37	5	0.016
	Medium	52.0			104			156		

*Periods of immersion were: in 50% SW, 8–10 hours; in 100% SW, 24 hours; in 150% SW, 8–10 hours.

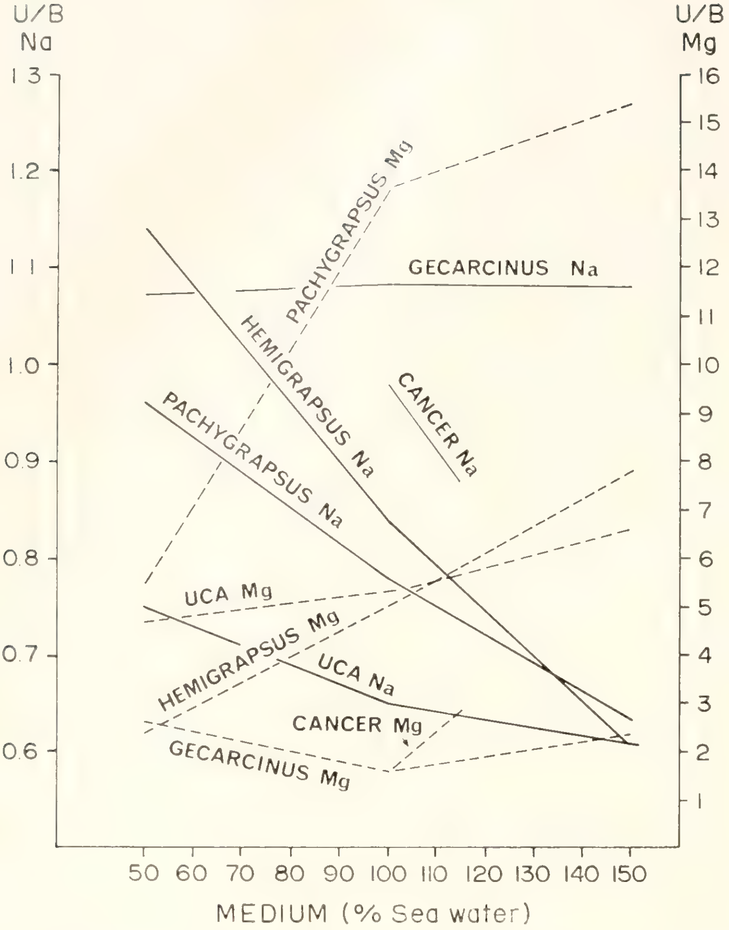


FIGURE 2. Comparison of Mg and Na U/B values for five species of crabs immersed in different concentrations of sea water. Curves are estimated from mean values in Table IV for *Cancer;* Table V for *Hemigrapsus;* Table VI for *Uca* and Table VII for *Gecarcinus.* Curves for *Pachygrapsus* are estimated from values given by Gross (1959). Solid line represents Na ratio; broken line, Mg ratio. Note different scales for Mg and Na.

treatments ($P < 0.001$). This phenomenon is illustrated in Figure 2 where also are shown the mean U/B values for Mg. Here it can be seen that for *Cancer, Hemigrapsus, Pachygrapsus* and *Uca,* the Na U/B values decreased with increasing salinity, but the U/B values for Mg increased. Yet, again, the Mg U/B value for *Gecarcinus* showed no trend with the salinity of the external medium. It has been demonstrated previously that the U/B values for both Na and Mg remain constant for *Gecarcinus* in a terrestrial situation regardless of the salinity of the available water (Gross, 1963a).

It is also important to examine the magnitudes of the U/B values for Na and Mg. Thus, with respect to Na this ratio averaged less than unity for all treatments in the cases of *Cancer, Pachygrapsus* and *Uca*. The mean U/B values for the three species were significantly different from unity ($P < 0.01$) for all treatments except *Cancer* in 100% sea water and *Pachygrapsus* in 50% sea water. The mean U/B values were significantly less than one for *Hemigrapsus* in 100% and 150% sea water ($P < 0.01$) and significantly greater than unity in 50% sea water ($P < 0.01$). On the other hand *Gecarcinus* demonstrated mean U/B values for Na which were significantly above unity ($P < 0.01$) for all treatments (Table VII).

The U/B values for Mg might suggest the relative role of the antennary gland in the regulation of this cation. Yet, *Pachygrapsus* showed the highest U/B

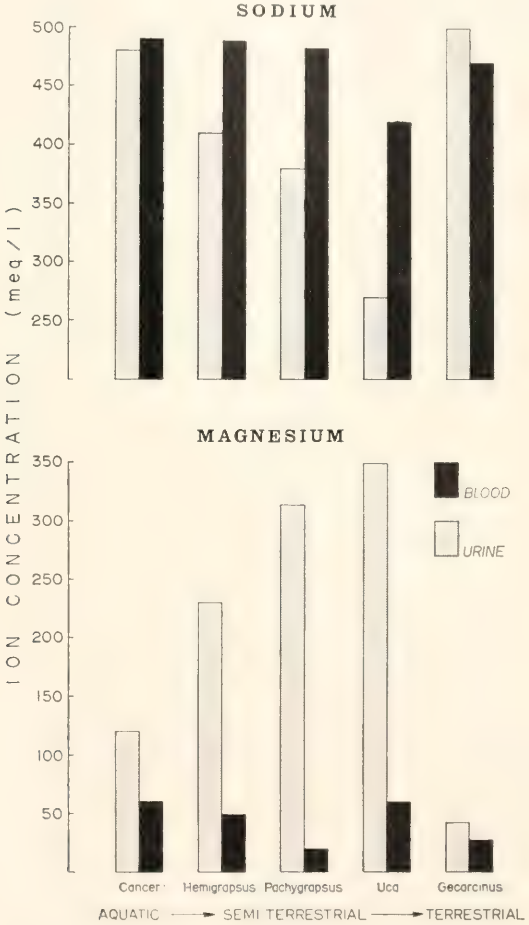


FIGURE 3. Concentrations of Na and Mg in blood and urine of crabs showing different degrees of terrestrialness. Height of bar represents mean ion concentration (meq./l.) of animals immersed in 100% sea water. Solid bar = blood; stippled bar = urine. Values for *Cancer* taken from Table IV; for *Hemigrapsus,* Table V; for *Uca,* Table VI and for *Gecarcinus,* Table VII. Values for *Pachygrapsus* are given by Gross (1959).

ratios for Mg (Fig. 2), but not the highest urine Mg concentrations (Fig. 3), and *Uca* demonstrated the highest urine Mg concentrations, moderate Mg U/B values and relatively high blood Mg concentrations (Table VI; Figs. 2 and 3).

Inasmuch as this ratio was lower for *Gecarcinus* immersed in 150% sea water (2.37), than in the weak Mg regulator, *Cancer,* immersed in 115% sea water (2.82), it is strongly suggestive that the antennary glands of *Gecarcinus* are relatively unimportant as Mg regulators. Still *Gecarcinus* maintained low blood Mg despite low Mg U/B values and low urine Mg concentrations, indicating that the inability of the antennary gland to concentrate Mg does not preclude strong Mg regulation by the animal. However, the response shown by *Uca* illustrates that the ability of the antennary gland to concentrate Mg does not assure strong Mg regulation by the animal.

Figure 3 compares Na and Mg regulation in five species of crabs immersed in 100% sea water, as related to their degree of terrestrialness. This treatment affords the most valid one for comparison because all species except *Gecarcinus* had been exposed to approximately normal sea water in their natural environments and in the laboratory for indefinite periods. The terrestrial *Gecarcinus* was immersed in 100% sea water for 24 hours. Thus, blood and urine Na seemed to decrease with increasing terrestrialness with the exception of *Gecarcinus,* the most terrestrial species. Also, with the exception of *Gecarcinus* urine Na was less concentrated than blood Na. With regard to Mg regulation, Figure 3 shows no correlation between blood Mg concentration and degree of terrestrialness. In fact, *Cancer* and *Uca* had the same average blood Mg concentrations (61 meq./l.). On the other hand, with respect to the ability of the antennary glands to concentrate Mg in the urine there was a trend with increasing terrestrialness, but, again, with the dramatic exception of *Gecarcinus,* which had the lowest urine Mg of all crabs studied. This suggests, of course, that low blood Mg in *Gecarcinus* is regulated by means other than the antennary gland.

It could be argued that the antennary glands of *Gecarcinus* are capable of concentrating Mg, but the animal is impermeable to this ion. In an exploratory experiment, sufficient $MgCl_2$ was injected into the blood space of three specimens to elevate the blood Mg about three-fold; still after 6 hours in every case, the U/B value for Mg was less than unity and the maximum Mg urine concentration was only 65 meq./l., which is far less than found in any of the other species immersed in normal sea water. Also, after this treatment all Na U/B values remained above unity. However, even in *Pachygrapsus* the urine Mg concentration is somewhat independent of the blood Mg concentration and directly independent of the influx of this ion from the external medium (Gross and Marshall, 1960).

Cancer also seems to concentrate urine Mg somewhat independently of Mg influx. In Table IV, Column D, it can be seen that when this crab was immersed for four hours in artificial sea water which had the osmotic concentration of 100% sea water but from which Mg has been deleted (Na was substituted for the deleted Mg to achieve the appropriate osmotic concentration), the average urine Mg concentration did not diminish from that the crab had in 100% natural sea water. This suggests, as it did for *Pachygrapsus* (Gross and Marshall, 1960), that the concentration of urine Mg is effected by the direction and magnitude of the water fluxes. However, it will also be noted (Table IV, Column C) that when *Cancer* was immersed for about 18 hours in artificial sea water which was equal to 100% sea water in osmotic concentration but which contained half again as much Mg (156 meq./l.) both urine and blood Mg increased significantly in concentration on the average over that which *Cancer* had in 100% natural sea water ($P < 0.01$). Therefore, while Mg regulation by the antennary gland of *Cancer* seems to be somewhat independent of Mg influx over a brief period, high Mg levels in the medium are reflected in the urine concentrations. Such was not the case for *Pachygrapsus,* whose urine Mg concentration seems to be dictated by the osmotic concentration of the external medium regardless of abnormally high or low Mg concentrations in that medium (Gross and Marshall, 1960).

It might be observed that when the medium Mg was abnormally high, the urine and blood Na of *Cancer* was lower than normal (Table IV, Column C). However, in the artificial medium containing high Mg, Na was reduced to achieve the appropriate osmotic concentration. It is believed, therefore, that the reduced Na concentration in blood and urine merely reflected the reduced concentration in the medium.

Exploratory experiments demonstrated that *Hemigrapsus* also can form a urine highly concentrated in Mg when immersed in a Mg-free medium.

Discussion

By examining the osmotic and ionic regulatory ability in a series of crabs showing different degrees of terrestrialness an attempt has been made to reveal some physiological trends which may have taken place during the evolutionary invasion of land from a marine environment. Yet it is not feasible to conduct immersion experiments on the subject species with precisely comparable conditions. For example, the natural ambient temperatures are considerably higher for *Grapsus* and *Ocypode* than for *Pachygrapsus* and *Hemigrapsus.* Thus, the question is posed as to whether it is more valid to study the different species at a common temperature or at the temperatures to which they are adapted in nature. The latter was chosen for this study because of the ecological emphasis of the investigation. Then, too, variation in size between the species doubtless influences the time required to reach osmotic equilibrium when immersed in a stress medium. Yet, variations in physical permeability would also influence the rate at which equilibrium is attained and this information is not available. Neither is information available on rates of acclimation to osmotic stress in any of these crabs except *Hemigrapsus* (Gross, 1963b). Also, objections could be raised to the longer period of immersion in stress media for *Uca* over the other species. However, exploratory studies showed that *Uca* probably attains osmotic equilibrium within 24 hours. Besides, even after 48 hours' immersion, *Uca,* next to *Ocypode,* is the most powerful osmotic regulator (Fig. 1). Of course, studies of all species immersed in 100% sea water, a medium to which all crabs except *Gecarcinus* had been exposed indefinitely, are most comparable. Perhaps the greatest objection would be raised to the incomplete and brief periods of immersion of *Gecarcinus* in stressed media, but intolerance to immersion permitted no alternative.

Despite these acknowledged objections, however, there are salient characteristics which are not obscured by these limitations. For example, there is a correlation between terrestrialness and hypoosmotic regulation. Although amphibious crabs show strong ability to concentrate Mg in their urine, there seems to be no correlation between Mg regulation and hypoosmotic regulation. For example, *Uca* is one of the most terrestrial crabs as well as one of the strongest hyporegulators (Fig. 1), but it can regulate its blood Mg no lower in concentration than the aquatic, nonregulator, *Cancer* (Fig. 3).

There is evidence that in all subject species of this study the blood and urine are isotonic with each other. On the other hand, in all species except *Gecarcinus,* the antennary glands concentrate Mg at the expense of Na. This, of course, permits isotonicity of urine and blood. The question should be asked, however, as to the physiological significance of high Mg concentrations in the urine. Surely the concentration of Mg in the urine does not assure low blood Mg (*e.g., Uca*) and low Mg concentrations in the urine do not mean high Mg concentration in the blood (*e.g., Gecarcinus*).

It is particularly interesting that the apparent trend toward higher urine Mg concentrations, among the more terrestrial amphibious crabs, collapses when the extreme land crab *Gecarcinus* is considered. The distinct difference between this land crab and the other species with respect to the handling of Na and Mg by the antennary glands suggests fundamentally different mechanisms. Moreover, it suggests a different pathway to the land habit than may be incipient in the amphibious crabs examined. That is, high urine Mg may reflect a response to conditions of hypersaline coastal lagoons. However, a capacity for *Gecarcinus* to hyporegulate could imply an ancestral exposure to hypersalinities. The inability of this crab to concentrate Mg in the urine and still maintain low blood Mg suggests an extra-renal mechanism for regulating this cation. Now *Gecarcinus* has a marine, free-swimming larva and thus is bound to the sea. Yet adult stages of this crab are intolerant to sea water (Gross, 1963a). It may be that the capacity of the antennary glands to concentrate Mg has been lost secondarily as has the tolerance to sea water. This then invites the study of immature stages of *Gecarcinus lateralis,* as well as the adult stages of some of the less terrestrial members of the Gecarcinidae. *Cardisoma guanhumi,* for example, is quite terrestrial, but habitually immerses itself in sea water or fresh water (Feliciano, 1962; Herreid and Gifford, 1963). Already, DeLeersnyder and Hoestlandt (1963) give evidence suggesting that *Cardisoma armatum* concentrates Mg in its urine when immersed in sea water.

Gross (1961) suggested that coastal lagoons afforded ideal sites for the evolution of land crabs. *Uca crenulata* is found in back bay regions and lagoons where high salinities would be expected, but not where low salinities would be common. However, in the summer of 1963, the author observed fiddler crabs (*Uca*) burrowed in the shores of the shallow waters of Long Key, Florida, where they were immersed in 170% sea water. During a torrential rainstorm, these same crabs were subjected to the run-off of fresh water and one hour later were entering burrows where the salinity was 17% sea water. It is obvious, then, that the marine shallows of tropical regions afford selective pressures favoring hypo- and hyperosmotic regulation. High water temperatures in these regions then might favor movements onto land. Perhaps the genus *Uca* evolved its terrestrial habits in such a situation. Verwey (1930) and Edney (1961) consider temperature and water problems in tropical fiddler crabs.

There is evidence suggesting three different types of responses in the brachyuran crabs with respect to the regulation of Mg by the antennary glands. First, *Cancer*, an aquatic crab, is capable of maintaining high urine Mg concentrations when exposed to a Mg-free medium for four hours. This might not be a sufficient period to permit an observable reduction in the urine Mg concentration. However, the animals became weak and died after longer exposures, and it is therefore believed that physiologically significant quantities of Mg were lost from the crab during that four-hour period. Assuming this to be so, *Cancer* concentrates its urine Mg somewhat independently of the Mg influx, *i.e.*, according to the osmotic concentration of the medium. Yet when *Cancer* is exposed to media containing abnormally high Mg concentrations, the antennary glands respond by concentrating Mg in the urine. Webb (1940) reported that *Carcinus* responds in a similar manner when the Mg of the external medium was increased.

The second type of response is that demonstrated by the amphibious *Pachygrapsus* which concentrates its urine Mg according to the osmotic concentration of the external medium and independently of the Mg influx. That is, the concentration of urine Mg in this animal depends on the osmotic concentration of the external medium, regardless of the amounts of Mg in that medium (Gross and Marshall, 1960). Of course, in a Mg-free medium, the prolonged exposure will exhaust the crab's Mg stores, and the urine Mg eventually will diminish.

The third type of response is that shown by the land crab, *Gecarcinus,* in which the antennary glands seem incapable of strongly concentrating urine Mg under any of the conditions examined in the present investigation. The U/B values for Mg, which are low but always greater than unity (except when injected with Mg) in this crab, might merely indicate water reabsorption from the primary urine rather than secretion of Mg.

The adaptive value of osmotic regulation for the land habit in crabs remains in question. Certainly it represents a degree of homeostasis which would be anticipated in animals exposed to an environment of variable stress. However, it is difficult to assign a specific function for the osmoregulatory mechanism of crabs when they are not immersed in water (Gross, 1955). On the other hand, amphibious and terrestrial crabs are commonly found in regions of fluctuating salinities such as mentioned above, and it may well be that selective pressures which encouraged the land habit were quite independent of, but imposed simultaneously with those favoring hypo- and hyperosmotic regulation.

Verwey (1957) suggests a correlation between varying environmental salinities and hypoosmotic regulation, pointing out that many purely aquatic crustaceans are capable of maintaining blood concentrations below those of the medium. He also states that many semi-terrestrial crabs cannot hyporegulate, but he gives no examples. Now the author is unfamiliar with any such amphibious crabs, but would be surprised, indeed, if this were not the case among the terrestrial potomonids which have invaded the land *via* the fresh water route. On the other hand, attention should be called to the case of *Hemigrapsus oregonensis* which, on the basis of laboratory studies, previously was considered incapable of hyporegulation (Jones, 1941), but later found living in a hypersaline lagoon and maintaining its blood quite hypotonic to a medium of 170% sea water (Gross, 1961). This case, of course, invites re-examination of other crabs presently considered incapable of hyporegulation.

No claim is made that hypoosmotic regulation is necessary for terrestrialness in crabs. Rather, attention is called to its common occurrence in crabs showing degrees of the terrestrial habit. While the functional significance of osmoregulatory mechanisms in land crabs should continue to be sought, perhaps consideration of the selective forces which favored the evolution of those mechanisms, as well as the selective pressures which encouraged the invasion of land, will lead to more meaningful explanations.

These studies were supported by a National Science Foundation Grant, G-18978. The work at the Eniwetok Marine Biological Laboratory was supported financially by the U. S. Atomic Energy Commission. I wish to express my gratitude to Messrs. John Armstrong and Ronald Capen for their able technical assistance.

SUMMARY

1. In order of their increasing terrestrialness the following seven species of crabs were studied with respect to their osmotic regulatory ability in aqueous media: the aquatic *Cancer antennarius, Hemigrapsus oregonensis,* the semi-terrestrial *Pachygrapsus crassipes, Grapsus grapsus, Ocypode ceratophthalma, Uca crenulata* and the terrestrial crab, *Gecarcinus lateralis.* Cation regulation was studied in all of the above except *Ocypode* and *Grapsus.*

2. All crabs examined except *Cancer* showed some degree of hypo- and hyperosmotic regulation. No correlation was found between the strength of hypoosmotic regulation and hyperosmotic regulation.

3. There is some correlation between degree of terrestrialness and the ability to hyporegulate. That is, the more terrestrial crabs are stronger hyporegulators than the more aquatic crabs.

4. The ratio, urine osmotic concentration/blood osmotic concentration (U/B), for *Hemigrapsus, Pachygrapsus, Grapsus, Uca* and *Ocypode* was essentially unity for all treatments. On the basis of cation concentrations in blood and urine, this seems to be true also for *Gecarcinus.* Therefore, there is no evidence that the antennary glands of any of these species are osmoregulatory in function.

5. There were no apparent trends with increasing terrestrialness with regard to the regulation of K.

6. Higher blood Ca concentrations were found in the more terrestrial crabs. Thus, when immersed in 100% sea water, the blood Ca of *Cancer* was 27.3 meq./l.; *Hemigrapsus,* 29.5 meq./l.; *Pachygrapsus,* 29.6 meq./l.; *Uca,* 39.3 meq./l. and for *Gecarcinus,* 44.2 meq./l.

7. Blood Ca remained relatively constant in all species even though the other cations were forced away from normal by osmotic stress.

8. The U/B values for K and Ca showed inconsistent patterns with respect to species and/or treatment. The significance of these values awaits information concerning K and Ca fluxes, as well as rates of urine flow.

9. The U/B values for Na decreased and those for Mg increased with increasing salinity of the external medium in *Cancer, Hemigrapsus, Pachygrapsus* and *Uca,* but there was no trend with respect to Na or Mg in *Gecarcinus.*

10. With the exception of *Gecarcinus,* the more terrestrial crabs concentrated Mg higher in the urine than did the more aquatic crabs.

11. Mg concentrations in the urine of *Gecarcinus* were the lowest of all crabs examined, suggesting the inability of the antennary glands in this species to regulate Mg. The low blood Mg of *Gecarcinus* immersed in sea water (27.8 meq./l.) indicates that low urine Mg does not preclude maintenance of low Mg in the blood.

12. The antennary glands of *Gecarcinus* showed little tendency to concentrate Mg even when the crab was injected with Mg.

13. The ability to concentrate Mg in the urine does not assure low Mg in the blood. Thus, the mean urine concentration for Mg in *Uca* immersed in sea water was 347 meq./l., yet its mean blood Mg was 61 meq./l., which is the same as that found for *Cancer* in which the urine Mg was only 102 meq./l. Since *Cancer* is aquatic and *Uca* quite terrestrial, there seems to be no correlation between Mg regulation in the blood and the land habit.

14. The response of *Gecarcinus* with respect to Na and Mg, which differs from other species studied, suggests a fundamentally different mechanism in the antennary glands and a pathway to terrestrialness which differs from that of the other crabs.

15. Three different types of response to Mg by the antennary glands of crabs are described.

16. The evolution of terrestrial crabs is discussed. It is suggested that osmotic regulation and terrestrialness may have arisen independently but simultaneously in regions of varying salinity and high water temperatures.

LITERATURE CITED

Bliss, D. E., 1962. The pericardial sacs of terrestrial Brachyura (Crustacea, Decapoda). *In:* Whittington, H. B. and Rolfe, W. D. I. (eds). Proceedings of the Conference on the Evolution of Crustacea. Cambridge, Mass.: Harvard University.

DeLeersnyder, M., and H. Hoestlandt, 1963. Premières données sur la régulation osmotique et la régulation ionique du crabe terrestre, *Cardisoma armatum* Herklots. *Cahiers de Biologie Marine,* **4**: 211–218.

Edney, E. B., 1960. Terrestrial adaptations. *In:* Waterman, T. H. (ed.). The Physiology of Crustacea, Vol. 1, 367: 393. New York: Academic Press.

Edney, E. B., 1961. The water and heat relationships of fiddler crabs (*Uca* spp.). *Trans. Roy. Soc. S. Afr.,* **36**: 71–91.

Feliciano, C., 1962. Notes on the biology and economic importance of the land crab *Cardisoma guanhumi,* Latreille of Puerto Rico. Special contribution, Institute of Marine Biology, University of Puerto Rico.

Flemister, L., 1958. Salt and water anatomy constancy and regulation in related crabs from marine and terrestrial habitats. *Biol. Bull.,* **115**: 189–200.

Flemister, L., and S. Flemister, 1951. Chloride ion regulation and oxygen consumption in the crab *Ocypode albicans* (Bosq). *Biol. Bull.,* **101**: 259–273.

Green, J. W., M. Harsch, L. Barr and C. L. Prosser, 1959. The regulation of water and salt by the fiddler crabs, *Uca pugnax* and *Uca pugilator. Biol. Bull.,* **116**: 76–78.

Gross, W. J., 1955. Aspects of osmotic regulation in crabs showing the terrestrial habit. *Amer. Nat.,* **89**: 205–222.

Gross, W. J., 1957. Analysis of response to osmotic stress in selected decapod Crustacea. *Biol. Bull.*, **112**: 43–62.

Gross, W. J., 1958. Potassium and sodium regulation in an intertidal crab. *Biol. Bull.*, **114**: 334–347.

Gross, W. J., 1959. The effect of osmotic stress on the ionic exchange of a shore crab. *Biol. Bull.*, **116**: 248–257.

Gross, W. J., 1961. Osmotic tolerance and regulation in crabs from a hypersaline lagoon. *Biol. Bull.*, **121**: 290–301.

Gross, W. J., 1963a. Cation and water balance in crabs showing the terrestrial habit. *Physiol. Zoöl.*, **36**: 312–324.

Gross, W. J., 1963b. Acclimation to hypersaline water in a crab. *Comp. Biochem. Physiol.*, **9**: 181–188.

Gross, W. J., 1964. Water balance in anomuran land crabs on a dry atoll. *Biol. Bull.*, **126**: 54–68.

Gross, W. J., and P. Holland, 1960. Water and ionic regulation in a terrestrial hermit crab. *Physiol. Zoöl.*, **33**: 21–28.

Gross, W. J., and L. Marshall, 1960. The influence of salinity on the magnesium and water fluxes of a crab. *Biol. Bull.*, **119**: 440–453.

Herreid, C. F., and C. A. Gifford, 1963. The burrow habitat of the land crab, *Cardisoma guanhumi* (Latreille). *Ecology*, **44**: 273–275.

Jones, L. L., 1941. Osmotic regulation in several crabs of the Pacific Coast of North America. *J. Cell. Comp. Physiol.*, **18**: 79–91.

Lockwood, A. P. M., 1962. The osmoregulation of Crustacea. *Biol. Rev.*, **37**: 257–305.

Pearse, A. S., 1934. Freezing points of bloods of certain littoral and estuarine animals. *Carnegie Inst. of Washington Papers from Tortugas Laboratory*, **28**: 93–102.

Prosser, C. L., J. W. Green and T. Chow, 1955. Ionic and osmotic concentrations in blood and urine of *Pachygrapsus crassipes* acclimated to different salinities. *Biol. Bull.*, **109**: 99–107.

Prosser, C. F., and F. A. Brown, Jr., 1961. Comparative Animal Physiology.. W. B. Saunders Co., Philadelphia (2nd ed.).

Rathbun, M. J., 1918. The grapsoid crabs of America. Bull. U. S. Nat. Mus., No. 97.

Verwey, J., 1930. Einiges aus die Biologie ostindischer Mangrove krabben. *Treubia.*, **12**: 167–261.

Verwey, J., 1957. A plea for the study of temperature influence on osmotic regulation. *Ann. Biol.*, **33**: 129–149.

Webb, D. A., 1940. Ionic regulation in *Carcinus maenas*. *Proc. Roy. Soc. London, Ser. B*, **129**: 107–136.

DONOR–HOST CELL INTERACTION IN HOMOLOGOUS SPLENOMEGALY IN THE CHICK EMBRYO

A. M. MUN AND E. R. BURNS[1]

Department of Zoology, University of Maine, Orono, Maine

The initial step in the splenomegaly observed in the chick embryo following the implantation of a piece of adult chicken spleen on the chorioallantoic membrane (CAM) involves a migration of donor cells into the spleen and other organs of the host, as was first inferred from the serial transfer studies by Simonsen (1957), and Ebert (1957). When either the blood or cells of the enlarged spleen from the first host are injected into or grafted to a new host, a similar enlargement of the spleen in the secondary host is obtained.

Direct evidence of this migration and subsequent colonization of donor cells in the host spleen was provided by Biggs and Payne (1959). Four days after the intravenous injection of cockerel blood into 14-day-old chick embryos, they were able to identify male cells in the enlarged spleens of female embryos. More recently, Becker *et al.* (1963) were also able to demonstrate the presence of donor cells containing a distinctive chromosome marker (T6) in large white clusters of cells or colonies in the spleens of heavily irradiated (900–1000 rads) mice. These macroscopic colonies appear to be derived exclusively from the proliferative activity of "stem" cells found in mouse hematopoietic tissues, as well as fetal liver. When highly inbred strains of mice were used, an "inhibition" rather than a stimulation of response was obtained, which was difficult to explain as either an immunologic "graft *vs.* host" or "host *vs.* graft" reaction (McCulloch and Till, 1963). In the chick, on the other hand, the resulting host spleen enlargement appears to be mediated by the migration, colonization, and extensive immunologic donor-host cell interaction in the host spleen.

In autoradiographic studies using tritiated thymidine, although distinctly labeled cells were found in the adult spleen graft on the CAM, few labeled cells were detected in the host spleen (Mun *et al.*, 1962). This observation argues against a massive migration of donor cells into the host spleen.

The rate of migration of these donor cells appears to be rapid. Mun *et al.* (1962) observed that as early as two days after a piece of adult chicken spleen was implanted, when the host spleen was then transferred to the CAM of a new host, although no enlargement of the embryonic spleen was detectable, it was able to elicit splenomegaly in the new hosts. Grafts of chick embryo spleen implanted approximately 1 cm. away from the adult spleen graft, when transferred to the CAM of new hosts, were also able to stimulate growth.

Within the host spleen, some of the invading adult cells proceed to launch an extensive "immunologic" attack. The immunological basis of this reaction has been well established. Danchakoff (1918) noted that embryonic spleen tissue was not able to stimulate growth. This ability was gradually developed in hatched chicks from the fifth day to the third week (Solomon, 1961; Mun *et al.*, 1962). Studies using highly inbred lines of chickens support the hypothesis that the splenic enlargement is due in part to the reaction of adult donor cells against the foreign antigens encountered in the host (see reviews of Billingham, 1959, and Ebert and DeLanney, 1960).

---

[1] NSF Summer Fellowship for Graduate Teaching Assistant. Present address: Department of Anatomy, School of Medicine, Tulane University, New Orleans 25, La.

The steps immediately following this immunologic attack are not quite as clear. Do the invading donor cells, having been stimulated by the host antigens, proceed to proliferate and form antibodies and eventually destroy or replace the native cell population (Simonsen, p. 449, 1957)? Or, do the donor cells, without undergoing any further proliferation, stimulate the *host* cells to divide? Or, both?

In the same study described above, Biggs and Payne (1959) found that, of the 170 cells examined in the enlarged spleen of female embryos, 75 were male cells and 95 were female cells. Thus, an appreciable portion of the splenic enlargement was provided by the host. Because a ratio of approximately 1:1 was observed in spleens enlarged five- to twenty-fold, this observation suggests not only that an appreciable portion of the splenic enlargement is provided by the host, but also that considerable proliferation of both donor and embryonic host cells is involved.

DeLanney *et al.* (1962) also observed a host response in the adult spleen graft on the chorioallantoic membrane. Cytological studies of the graft, at one- to two-day intervals, from the 1st to 10th day post-implantation, revealed dense aggregations of hemocytoblasts in the adjacent membranes by the 4th day post-implantation. Granulocytes, usually associated with an immunological type of response, were found in significant concentrations by the 5th day post-implantation.

In order to analyze the mechanism of stimulation of growth in the embryo, we may first inquire: Is the proliferation of embryonic cells necessary to produce the observed enlargement? Or is the proliferation of adult donor cells sufficient? What is the role of the adult donor cells in this stimulation? Is active proliferation of donor cells necessary to bring about the stimulation of embryonic components? The main objective of this study is to elucidate the role of the embryonic or host component in this splenomegaly reaction. We shall first examine the sufficiency and/or necessity of embryonic cells in this phenomenon and then explore the mechanism of this stimulation to proliferation (Mun, 1963).

Materials and Methods

The chorioallantoic membrane grafting technique (Willier, 1924; Hamburger, 1960) was used throughout this study. A quadrilateral window 1 × 1 cm. was cut in the shell with a fine-toothed hacksaw blade (Milford Midget, HS32T $\frac{1}{4}$ × 0.014 inch without set, Henry G. Thompson & Son Company, New Haven, Connecticut). A piece of tissue approximately 1 × 1 × 2 mm. and weighing 5 to 10 mg. was placed on the exposed membrane. The shell membrane and shell were replaced and sealed with paraffin. The eggs were returned to the incubator with the pointed end down.

5-Fluorouracil (5FU) was provided by Hoffmann-La Roche Laboratories, Nutley, N. J. Approximately 0.35 to 0.40 mg. of 5FU in 0.07- to 0.08-ml. volumes were injected into a blood vessel in the chorioallantoic membrane in the direction of flow (Terasaki and Cannon, 1957). A one-ml. syringe and a 30-gauge needle with a 22° bend was used. The syringe was held in place with a thermometer clamp and the egg was moved into position by means of a Cenco-Lerner Micro Labjack (Central Scientific Co., Chicago, Ill.).

The host spleens were removed on the 17th or 18th day of incubation and weighed to the nearest 0.2 mg. The graft on the CAM was graded according to the degree of incorporation and vascularization (Mun, Kosin and Sato, 1959). The embryo was drained, blotted on paper, and weighed to the nearest 0.1 gram.

I. THE ROLE OF THE HOST COMPONENT IN THE SPLENOMEGALY REACTION

To determine whether embryonic or host cell proliferation plays a sufficient and/or necessary role in this splenomegaly reaction, two experiments were conducted in which two different strains of chickens were used: (1) a highly inbred line (Line 7, Regional Poultry Research Laboratory, East Lansing, Michigan) with a coefficient of inbreeding of greater than 95%, and (2) a commercial strain (Queens Line, Arbor Acres Hatchery, Skowhegan, Maine).

TABLE I

The effect of adult spleens from Line 7 chickens (A7) on the mean spleen weight of Line 7 (E7) and Queens Line (QL) host embryos

Adult donor	First host embryo		Second host embryo		
Line	Line	Mean spleen wt. (mg. ± S.D.)	Line	Mean spleen wt. (mg. ± S.D.)	Group
A7	E7	8.91 ± 4.66 (18)	E7	10.80 ± 1.55 (15)	1
			QL	24.57 ± 20.10 (25)*	2
	QL	68.41 ± 33.18 (19)	QL	70.64 ± 36.16 (33)**	3
			E7	18.32 ± 9.74 (29)**	4
Chick Ringer saline	QL	8.72 ± 1.55 (9)	QL	10.28 ± 4.20 (8)	5
Chick Ringer saline	E7	8.76 ± 1.30 (8)	E7	11.33 ± 0.88 (6)	6

*($p<0.05$).
**($p<0.01$).
Figures in parentheses indicate number of cases.

A piece of adult spleen from an adult Line 7 chicken was implanted on the CAM of 10-day-old Line 7 and Queens Line embryos. After 7 more days of incubation, the host spleens were removed, weighed, then cut in half or in 5 mg. pieces, and transferred to the CAM of new 10-day-old Line 7 and Queens Line hosts. After seven more days of incubation, the spleens from the second hosts were removed and weighed. Thus four groups or series were established.

As may be seen in Table I, when the 1st and 2nd host embryos were derived from the same line as the adult donor (Line 7), the host spleens did not increase in size

(Group 1). However, when the second host was derived from a line different from that of both the adult donor or the first host, we obtained an increase in the weight of the host spleen (Group 2). Because donor spleen tissues from either 17-day-old Line 7 (Group 6) or Queens Line embryos (Group 5) were not able to stimulate growth, we may suggest that a sufficient number of competent adult Line 7 cells was probably transferred from the first host to the second host. These competent cells then proceeded to bring about the splenic enlargement observed in the second host (Group 2).

We may also note that, although an enlargement of the second host spleen was obtained, the spleen of the first host was not enlarged in Group 2. This observation suggests that the number of cells involved in the "graft *vs.* host" reaction is not large, which agrees with previous observations in which we failed to detect a large number of H^3 thymidine-labeled donor cells in the host spleen (Mun *et al.*, 1962).

In the third group, when both the first and second hosts were derived from a line different from that of the adult donor, both hosts' spleens were enlarged. However, in the fourth group, although the second host was derived from the same line as the adult donor, we also obtained a significant ($P < 0.01$) increase in size of the host spleen, suggesting that both adult donor *and* embryo cells proliferate in the first host and are transferred to the second host in sufficient quantities to elicit splenomegaly.

Whether the proliferation of host cells was necessary in this phenomenon may be ascertained by exploring the consequences of their inactivation or elimination. Mun, Kosin and Sato (1959) have previously studied the effects of inactivation of adult chicken spleen tissue by x-irradiation. With increasing dosage of x-irradiation the ability of the donor tissue to elicit splenomegaly was gradually diminished. Heating or freezing the adult spleen tissue before implantation on the CAM also removed its ability to stimulate growth. This suggested that viable adult cells capable of undergoing mitosis were probably necessary to elicit growth.

In an attempt to inactivate the embryonic component, 5-fluorouracil (5FU) was injected intravenously into chick embryos following the implantation of adult chicken spleen on the chorioallantoic membrane.

II. The Effect of 5-Fluorouracil on the Splenomegaly Reaction [2]

5-Fluorouracil has been found to be a highly effective inhibitor in many rapidly growing systems, including regeneration of the liver after partial hepatectomy, growth of the seminal vesicle induced by testosterone, growth of epiphyseal cartilage induced by growth hormone, and foetal rat growth (Paschkis *et al.*, 1959). It also inhibits the growth of mouse and rat tumors (Heidelberger *et al.*, 1957a, 1957b, 1958; Law, 1958), Rous sarcoma (Bather, 1960) and many human carcinomas (Ansfield and Curreri, 1959; Ansfield, Schroeder and Curreri, 1962). Karnofsky, Murphy and Lacon (1958) injected 5FU into the yolk sac of 8-day-old chick embryos and observed feather inhibition and edema. An LD_{50} of 1.0 mg. 5FU/egg at 8 days of incubation was established.

---

[2] From work reported in a thesis by Mr. E. R. Burns, submitted to the Faculty of the Graduate School in partial fulfillment of requirements for the degree, Master of Science, in the Department of Zoology, University of Maine, June, 1963.

To determine the effect of 5FU on the splenomegaly phenomenon, 0.25 mg. to 0.75 mg. of 5FU was injected intravenously into 11½-day-old chick embryos which had previously received chorioallantoic membrane grafts of adult chicken spleen on the tenth day of incubation. The 1½-day interval between graft and injection was judged to be adequate to permit a sufficient number of donor cells to migrate to the host spleen (Mun *et al.*, 1962).

When the host spleen was recovered on the 17th day of incubation, a dose of 0.50 mg. 5FU was found to be sufficient to inhibit the splenomegaly reaction (Table II). The mean spleen weights of the 0.50-mg. and 0.75-mg. 5FU groups were statistically different from the control groups receiving uracil, but not different from the mean spleen weight of sham operated embryos. In severely inhibited embryos, feather inhibition, edema, and other anomalies were observed.

TABLE II

The effects of 5-fluorouracil (5FU) 1½ and 3½ days after the implantation of adult spleen

Number of days after implantation	Treatment	No. cases	Mean spleen wt. (mg. ± S. D.)	Mean body wt. (mg. ± S.D.)
1½	0.25 mg. 5 FU	12	62.20 ± 42.14	14.24 ± 1.85
	0.25 mg. uracil	4	81.85 ± 48.16	14.70 ± 2.60
	0.50 mg. 5FU	23	11.04 ± 9.48	11.65 ± 1.78
	0.50 mg. uracil	9	32.38 ± 16.76**	13.98 ± 1.50
	0.75 mg. 5FU	54	6.66 ± 4.35	10.19 ± 1.00
	0.75 mg. uracil	18	30.44 ± 15.50**	13.51 ± 2.16*
	0.1 cc. chick Ringer	13	8.52 ± 1.25	16.49 ± 1.64
3½	0.25 mg. 5FU	10	34.88 ± 11.60	13.17 ± 1.18
	0.50 mg. 5FU	7	24.82 ± 25.56	13.52 ± 1.69
	0.75 mg. 5FU	42	11.04 ± 4.66	13.71 ± 1.86
	0.75 mg. uracil	29	46.91 ± 19.04**	15.08 ± 1.55
	1.50 mg. 5FU	10	5.53 ± 1.34	13.20 ± 1.20
	0.1 cc. chick Ringer	7	10.57 ± 2.32	16.94 ± 1.42

*($p<0.05$).
**($P<0.01$).

When 5FU was injected into 13½-day-old chick embryos, 3½ days after the implantation of adult chicken spleen, 0.75 mg. 5FU was found to be sufficient to inhibit the splenomegaly. However, the teratogenic effect of the drug was greatly reduced or absent.

This inhibition of the splenomegaly reaction may be due to the action of the drug on the donor or host cells, or both. However, when the inhibited or unenlarged host spleen was transferred to the CAM of a new host, it was able to elicit splenomegaly (Table III). This observation revealed that although proliferation of the donor cells was inhibited, viable and competent adult donor cells were still present in the host spleen.

III. THE STIMULATION OF EMBRYONIC OR HOST COMPONENT IN THE SPLENOMEGALY REACTION

We now ask: why was a stimulation of growth not obtained in the spleen of the first host? Was it due to the inhibitory action of the drug on the proliferation of the adult cells? Or was it due to the blocking action of 5FU on the proliferation of embryonic host cells following stimulation by the adult donor cells? Or both? If the ratio of donor to host cells in the enlarged spleen is approximately 1:1, as observed by Biggs and Payne (1959), then there is probably some inhibition of the mitotic activity of the adult donor cells. This block may have been removed upon transfer of the host spleen to the CAM of a new host. However, are these inhibited donor cells in the host spleen capable of stimulating embryonic cells?

TABLE III

The effect of the transfer of embryonic and adult spleen grafts to new embryonic hosts

Days after implantation	First host			Second host	
	Treatment	No. cases	Mean spleen wt. (mg. ± S.D.)	No. cases	Mean spleen wt. (mg. ± S.D.)
1½	0.75 mg. 5FU	17	6.67 ± 4.31	14 spleens	28.24 ± 13.76
	0.75 mg. uracil	3	21.26 ± 5.38	3 spleens	40.27 ± 13.85
	0.1 cc. chick Ringer	6	9.82 ± 1.44	3 spleens	8.90 ± 0.60
3½	0.75 mg. 5FU	9	10.28 ± 2.24	12 grafts 9 spleens	52.93 ± 34.73 48.97 ± 25.63
	0.75 mg. uracil	12	34.02 ± 15.40	7 grafts 4 spleens	55.25 ± 35.37 95.05 ± 51.16
	0.1 cc. chick Ringer	2	11.44 ± 2.27	3 spleens	10.26 ± 0.81

To examine this possibility, as well as to establish the role of the embryonic component in this splenomegaly reaction, fresh embryonic spleen tissue was added to the same host which had previously received a chorioallantoic graft of adult chicken spleen and an intravenous injection of 5-fluorouracil. If embryonic cells are involved in this splenomegaly reaction, then a definite response to the second embryonic spleen graft should ensue, even though active proliferation of the donor component was curtailed.

The following experimental procedure was adopted:

A piece of adult chicken spleen was implanted on the CAM on the 9th day of incubation. Two days later, on the 11th day of incubation, 0.35 mg./0.07 ml. of 5FU was injected intravenously. Three days later on the 14th day of incubation, a piece of 17-day-old chick embryo spleen was implanted on the CAM approximately 1 cm. away from the first graft.

On the 18th day of incubation, the shell was removed, together with the adhering chorioallantoic membrane in which both the first and second grafts were imbedded. The lengths and widths of the two grafts were measured with a pair of vernier calipers to the nearest 0.1 mm. The grafts were then cut out, trimmed of all excess membranes and weighed to the nearest 0.2 mg. The host spleen was removed and weighed to the nearest 0.2 mg. The embryos were separated into 4 groups according to the degree of abnormality induced:

Group I. Embryos appeared normal.

Group II. Body weight appeared normal but feathers were approximately one-half of normal length, giving embryos a fuzzy appearance.

Group III. Body weight was more than 3 grams below normal. Feather formation was inhibited by at least two stages (Hamburger and Hamilton, 1951).

Table IV

Effect of adult spleen (AS) and/or embryonic spleen (ESG_1) and 5-fluorouracil (5FU) on a second embryonic spleen graft (ESG_2)

Treatment			Mean host spleen wt. (mg. ± S.D.)	Mean ESG_2 wt. (mg. ± S.D.)
Day of incubation				
9-day	11-day	14-day		
AS + ESG_1	—	—	50.02 ± 18.99 (9)	
AS	5FU	—	16.50 ± 9.85 (10)	
AS + ESG_1	5FU	–	13.51 ± 4.22 (7)	
AS	5FU	ESG_2	9.85 ± 4.68 (28)*	34.71 ± 13.33 (28)**
ESG_1	5FU	ESG_2	6.96 ± 2.69 (16)	17.83 ± 7.44 (16)
AS	—	ESG_2	29.66 ± 15.17 (18)**	33.60 ± 14.50 (18)**
ESG_1	—	ESG_2	12.45 ± 2.89 (17)	20.55 ± 8.74 (16)
—	5FU	AS	6.73 ± 3.69 (24)	
—	—	AS	20.36 ± 22.12 (18)	

*($p < 0.05$).

**($P < 0.01$).

Figures in parentheses indicate number of cases.

Group IV. Body weight was more than 3 grams below normal. Feather formation was inhibited more than two stages. Other abnormalities were also present, including edema in the head and/or abdominal regions, twisted beaks, and curled toes.

Only embryos from Groups III and IV were included in the tabulations.

As may be seen in Table IV, an enlargement of the spleen was not obtained by adding a second embryonic spleen graft into hosts which had previously received an adult spleen graft and an injection of 5FU. However, the weight of the second embryonic spleen graft was found to be significantly greater ($P < 0.01$) than that implanted in an embryo which had previously received an embryonic spleen graft and an injection of 5FU. This observation reveals not only that viable and competent donor cells are still present in the adult spleen graft, but also that these cells are capable of migrating to the second embryonic spleen graft and stimulating growth there.

DISCUSSION

When the enlarged host spleen was transferred to the chorioallantoic membrane of a new host, it was able to elicit splenomegaly if the genetic strain of the second host was similarly different from that of the adult donor (Group 3, Table I). This suggests that the adult donor cells are capable of migrating to and invading the spleen of the embryonic hosts. Such a mechanism was inferred earlier in serial transfer studies of Simonsen (1957) and Ebert (1957), using non-inbred lines of chickens. On the other hand, when the second host was derived from the same inbred line as that of the adult donor but different from that of the first host, we also observed a stimulation (Group 4, Table I). This observation strongly suggests that embryonic spleen cells are also capable of being transferred to the spleen of the new host. Such a mechanism was inferred in a previous study in which embryonic spleen tissue was serially propagated in non-inbred embryos. After five or six transfers, a cumulative response was obtained, approximately paralleling the normal development in the chicken of the ability to elicit splenomegaly (Mun *et al.*, 1962). Because the embryonic host cells could only be transferred if they were present in adequate numbers, this observation may also lend support to the cytological observations of Biggs and Payne (1961), which indicated that both adult and embryonic host cells proliferated in the first host.

The splenomegaly reaction was clearly inhibited by 5-fluorouracil. Preliminary studies of histological sections of the inhibited spleen revealed a variety of chromosomal aberrations, suggesting that the focus of attack of the drug is upon the mitotic apparatus. Berger and Witkus (1962) found chromosomal elimination, binucleate cells and chromosomal fragmentation in *Allium cepa* root tips treated with 5-fluorouracil.

The effect of the drug on feather development was very striking. However, growth of the entire embryo appears to be affected, as judged by the stage of development (Hamburger and Hamilton, 1951) attained. When the drug was introduced on the 13½ to 14th day of incubation, its effect on growth as well as feather formation was greatly reduced or absent.

The resulting splenomegaly observed following the transfer of the 5FU-inhibited host spleens to new hosts suggests, however, that the action of the drug is not primarily on the ability of the adult donor cells to stimulate growth, but on the ability of either the adult donor cells or embryonic host cells, or both, to proliferate.

The second possibility was tested by the addition of fresh embryonic tissue to the 5FU-inhibited embryo. Because an enlargement of this second embryonic spleen graft was obtained, we may postulate that splenomegaly is due in part to the proliferation of host embryo cells following stimulation by the adult donor cells. Of course, we must assume that (1) competent adult cells in the initial graft are capable of migrating on the 14th day of incubation to the host spleen as well as to the second embryonic spleen graft in embryos injected with 5FU, and that (2) the transferred donor cells are then able to proliferate freely at either site.

Although we cannot exactly ascertain the mobility or activity of the adult donor cells on the 14th day of incubation, we can demonstrate that viable donor cells are probably already present in the host spleen because the reaction can still be transferred.

The size of the grafts on the chorioallantoic membrane may be influenced by the activity of the host cells. Tardent, Mun and Ebert (*cf.* Mun *et al.*, 1962) found labeled cells in the midst of an unlabeled adult chicken spleen graft on the CAM of a host previously injected with H^3 thymidine. Although cytological studies are not complete, gross examinations of second embryonic spleen grafts in hosts which received initially either embryo or adult spleen grafts reveal no edema or extreme thickening of the CAM.

The failure to obtain enlargement of the host spleen in 5FU-treated embryos following the addition of a second embryonic spleen graft on the 14th day of incubation is more difficult to explain, especially in the light of the first series of experiments which suggest that cells from enlarged embryonic spleens may be transferred to the spleen of the new host. Several possible explanations may be advanced. The first possibility is that the concentration of 5FU in the host spleen was still high enough to inhibit the activity of the transferred embryonic cells. This possibility, however, requires that the drug be maintained in higher concentrations in the host spleen than in the extra-embryonic membrane, or in other organs in the body, which may be unlikely. A second possible explanation is the failure of a sufficient number of embryonic cells to be transferred to the host spleen. As shown in Table IV, a striking enlargement of the host spleen is obtained when adult chicken spleen is implanted on the 10th day of incubation. However, when adult spleen was grafted on the 14th day of incubation, the stimulation was greatly reduced. This observation is in line with that of Solomon and Tucker (1963) who obtained splenomegaly when the adult spleen was implanted on the 8th, 9th, 11th and 13th day of incubation. Implantation on the 15th day of incubation produced only a slight increase in host spleen weight. The second embryonic spleen graft, on the other hand, was stimulated to grow by the adult donor cells, probably because it was well incorporated and contained a sufficient number of viable cells.

These evidences suggest strongly that the host spleen enlargement involves in part a stimulation of the host cells by the adult donor cells. From the work of Till *et al.* (1964) we must consider, in addition, the possibility of host "stem" cell as well as donor "stem" cell proliferation. The nature of the donor host cell interaction or the control mechanisms which could act to alter the activity of the stem cells remains to be explored.

The authors wish to acknowledge the advice and suggestions of Drs. B. R. Speicher and C. W. Major. We also wish to thank Dr. James D. Ebert for critical reading of the manuscript. A grateful acknowledgment is made to Dr. B. Winton, Director, and Dr. L. B. Crittenden, Regional Poultry Research Laboratory, East Lansing, Michigan, for providing invaluable inbred material. We wish to thank Mrs. Carolyn B. Johnston for technical assistance. This investigation was supported by grants from the National Science Foundation, G-22431, and Coe Research Fund, University of Maine.

SUMMARY

1. Adult chicken spleen from a highly inbred line (Line 7) was implanted on the chorioallantoic membrane of embryos from the same line. When the host spleen was transferred to the chorioallantoic membrane from a different strain, a

striking enlargement was obtained, which suggested that adult donor cells were transferred from the first host.

2. When the first host was derived from a different line, and the host spleen was then transferred to another host of the same line as the adult donor, a striking enlargement of the host spleen was observed. This suggests that both adult and host cells were transferred to the second host and also that proliferation of the host embryonic component may have occurred.

3. The splenomegaly phenomenon was inhibited by the intravenous injection of 5-fluorouracil following the implantation of adult chicken spleen. However, the unenlarged host spleen, when transferred to another host embryo, was able to elicit splenomegaly. This reveals the presence of viable and competent donor cells in the treated host spleen.

4. When a fresh piece of 17-day-old chick embryo spleen was added to the chorioallantoic membrane of an embryo which had previously received a graft of adult spleen and an injection of 5-fluorouracil, a significant enlargement of the second embryonic spleen graft was observed.

5. These observations suggest that the splenomegaly reaction is due in part to the proliferation of host embryo cells following stimulation by the adult donor cells.

LITERATURE CITED

Ansfield, F. J., and A. R. Curreri, 1959. Further clinical studies with 5-fluorouracil. *J. Nat. Can. Inst.,* **22**: 497–507.

Ansfield, F. J., J. M. Schroeder and A. R. Curreri, 1962. Five years clinical experience with 5-fluorouracil. *J. Amer. Med. Assoc.,* **181**: 295–299.

Bather, R., 1960. The use of young chicks and virus-induced tumors for testing chemotherapeutic agents. *Acta: Unio Intern. contra Cancrum,* **16**: 545–551.

Becker, A. J., E. A. McCulloch and J. E. Till, 1963. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature,* **197**: 452–454.

Berger, C. A., and E. R. Witkus, 1962. Cytological effects of 5-fluorouracil. *Exp. Cell Res.,* **27**: 346–350.

Biggs, P. M., and L. N. Payne, 1959. Cytological identification of proliferating donor cells in chick embryos injected with adult chicken blood. *Nature,* **184**: 1594.

Biggs, P. M., and L. N. Payne, 1961. Pathological changes following the inoculation of chick embryos with adult cells. I. Spleen cells. *Immunology,* **4**: 24–37.

Billingham, R. E., 1959. Reactions of grafts against their hosts. *Science,* **130**: 947–953.

Cock, A. G., and M. Simonsen, 1958. Immunological attack on newborn chickens by injected adult cells. *Immunology,* **1**: 103–110.

Danchakoff, V., 1918. Equivalence of different hematopoietic anlages (by method of stimulation of their stem cells). II. Grafts of adult spleen on the allantois and response of the allantoic tissues. *Amer. J. Anat.,* **24**: 127–189.

DeLanney, L. E., J. D. Ebert, C. M. Coffman and A. M. Mun, 1962. On the chick spleen: Origin; patterns of normal development and their experimental modification. *Carnegie Inst. Washington, Contrib. to Embryology,* **37**: 57–85.

Ebert, J. D., 1957. Annual Report of the Director of the Department of Embryology for 1956–1957. *Carnegie Inst. Wash. Year Book,* **56**: 297–356.

Ebert, J. D., and L. E. DeLanney, 1960. Ontogenesis of the immune response. *Nat. Cancer Inst. Monographs,* **2**: 73–111.

Hamburger, V., 1960. A Manual of Experimental Embryology. 221 pages. University of Chicago Press, Chicago, Illinois.

Hamburger, V., and H. L. Hamilton, 1951. A series of normal stages in the development of the chick embryo. *J. Morph.,* **88**: 49–92.

Heidelberger, C., N. K. Chaudhuri, P. Danneberg, D. Mooren and L. Griesgach, 1957a. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature,* **179:** 663–666.

Heidelberger, C., L. Bosch, N. K. Chaudhuri and P. Danneberg, 1957b. Mechanisms of action of 5-fluoro-pyrimidines. *Fed. Proc.,* **16:** 194.

Heidelberger, C., L. Griesgach, B. Montag, D. Mooren and O. Cruz, 1958. Studies on fluorinated pyrimidines: II. Effects on transplanted tumors. *Can. Res.,* **18:** 305–317.

Karnofsky, D. A., M. L. Murphy and C. R. Lacon, 1958. Comparative toxicologic and teratogenic effects of 5-fluoro-substituted pyrimidines in the chick embryo and pregnant rat. *Proc. Amer. Assoc. Can. Res.,* **2:** 312–313.

Law, L. W., 1958. The effect of fluorinated pyrimidines on neoplasms of the blood and blood-forming organs. *Proc. Amer. Assoc. Can. Res.,* **2:** 318–319.

McCulloch, E. A., and J. E. Till, 1963. Repression of colony-forming ability of C57BL hematopoietic cells transplanted into non-isologous host. *J. Cell. Comp. Physiol.,* **61:** 301–308.

Mun, A. M., 1963. Graft-host cell interaction in the splenomegaly reaction in the chick embryo. *Proceedings XVI International Congress of Zoology, Washington,* **2:** 223.

Mun, A. M., I. L. Kosin and I. Sato, 1959. Enhancement of growth of chick host spleens following chorio-allantoic membrane grafts of homologous tissues. *J. Embryol. Exp. Morph.,* **7:** 512–525.

Mun, A. M., P. Tardent, J. Errico, J. D. Ebert, L. E. DeLanney and T. S. Argyris, 1962. Analysis of the initial reaction in the sequence resulting in homologous splenomegaly in the chick embryo. *Biol. Bull.,* **123:** 366–387.

Paschkis, K. E., D. Bartuska, J. Zagerman, J. W. Goddard and A. Cantarow, 1959. Effect of 5-fluorouracil on noncancerous tissue growth. *Can. Res.,* **19:** 1196–1203.

Simonsen, M., 1957. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Pathologica et Microbiologica Scandinavica,* **40:** 480–500.

Solomon, J. B., 1961. The onset and maturation of the graft versus host reaction in chickens. *J. Embryol. Exp. Morph.,* **9:** 355–369.

Solomon, J. B., and D. F. Tucker, 1963. Immunological attack by adult cells in the developing chick embryo: Influence of the vascularity of the host spleen and of homograft rejection by the embryo on splenomegaly. *J. Embryol. Exp. Morph.,* **11:** 119–134.

Terasaki, P. I., and J. A. Cannon, 1957. A technic for cross-transfusion of blood in embryonic chicks and its effect upon hatchability. *Proc. Soc. Exp. Biol. Med.,* **94:** 103–107.

Till, J. E., E. A. McCulloch and L. Siminovitch, 1964. A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proc. Nat. Acad. Sci.,* **51:** 29–36.

Willier, B. H., 1924. The endocrine glands and the development of the chick. I. The effect of thyroid grafts. *Amer. J. Anat.,* **33:** 67–103.

ECHOLOCATION OF FLYING INSECTS BY THE BAT, CHILONYCTERIS PARNELLII

ALVIN NOVICK AND JUOZAS R. VAISNYS

*Department of Biology and Department of Engineering and Applied Science,
Yale University, New Haven, Connecticut 06520*

Some aspects of acoustic orientation have been observed and analyzed in 13 families of bats but only a few genera of the family Vespertilionidae have been studied extensively experimentally while they have been hunting insects or avoiding obstacles (Griffin, 1953, 1958; Grinnell and Griffin, 1958; Griffin, Webster and Michael, 1960; Cahlander, McCue and Webster, 1963). Griffin (1962) has reported more briefly some interesting observations on *Noctilio* and *Rhinolophus*. Pye (1960, 1961a, 1961b) and Kay (1961, 1962) have advanced hypotheses for a mechanism of echolocation based in part on the overlap in time (at the bat's ear) between orientation pulses and their echoes, when, because of the frequency modulated design of the orientation pulses produced by bats of several families (Griffin, 1958; Novick, 1958, 1962, 1963a), the bat could perceive a beat note. Depending upon the precision of discriminative perception and the bat's awareness of the frequency characteristics and the regularity of its output, such a system could be used for detecting the distance of an echoing object. These hypotheses have not been compatible with the observations on vespertilionids where the pulses shorten dramatically as the bat closes in on its prey, apparently in order to preclude overlap (Griffin, 1958; Griffin, Webster and Michael, 1960; Cahlander, McCue and Webster, 1963). Novick (1963b) reported that for the phyllostomatid bat, *Pteronotus,* pulse-echo overlaps appear to be characteristic and regular in their occurrence and degree during insect pursuits. These observations revived interest in the significance of pulse duration and pulse-echo interaction in echolocation in bats.

The orientation sounds of one individual *Chilonycteris parnellii mexicana* Miller (Phyllostomatidae) (Hall and Kelson, 1959) have been recorded while the bat was flying around a laboratory flight room and occasionally pursued and apparently captured common fruit flies, *Drosophila* sp. The orientation of *Chilonycteris* has previously been studied by Griffin and Novick (1955) and Novick (1963a). The bat referred to by these authors as *Chilonycteris rubiginosa* is apparently the same as *C. parnellii.* These bats are delicate in captivity but one individual captured in the Cueva del Salitre, Morelos, Mexico, in March, 1963, survived for six months in New Haven, feeding on mealworms and cockroaches. We are grateful to Drs. B. Villa-R. and W. Wimsatt for their help in capturing this bat, to the Institute of Biology of the National University of Mexico for the use of their facilities during part of this work and to the Mexican government for permission to capture and transport this bat. This work has been supported in part by the National Institute of Mental Health and by the Air Force Office of Scientific Research.

The bat sounds were recorded with a custom-made Granath microphone and a Precision Instrument PI-202 tape recorder running at 60 ips. We were only able to identify and analyze four complete hunting sequences of sufficient signal: noise ratio. These have been examined in detail from filmed oscillograph tracings and from Kay Electric Co. sound spectrographs.

The basic design of the orientation pulses of this species has been described elsewhere (Novick, 1963a). A typical pulse consists of a dominant component of about 32 kc accompanied by harmonics. The frequency is constant except for a momentary rise initially from about 30 kc and a terminal drop to about 28 kc. The initial rise in one "typical" pulse occupied about 0.5 msec.; the terminal drop occupied about 1.7 msec. of a 22.5-msec. pulse.

The present analysis deals chiefly with the significance of variations in pulse duration as seen in these records and with the calculated pulse-echo overlaps. As in the vespertilionids (Griffin, Webster and Michael, 1960) and *Pteronotus* (Novick, 1963b), these pursuits may be subdivided into search, approach and terminal phases (Fig. 1). The search phase cannot be distinguished as yet, in any bat, from its regular, cruising orientation sequence. Presumably during this phase, the bat has no knowledge of its future detection of an obstacle or a prey, or, at least, takes no observed behavioral notice of such. The approach phase begins, by definition in these analyses, at the point at which we first recognize that the bat is committed to a pursuit. In *Pteronotus* (Novick, 1963b), this point was recognized by the first obvious shortening of the pulse-to-pulse interval. In *C. parnellii,* the beginning of the approach phase is signaled by an increase in pulse duration (Fig. 2). During the approach phase, when the bat is presumably clarifying the position, nature, and velocity of its target and arranging its own flight path, systematic variations in pulse duration and/or pulse-to-pulse interval can be plotted. The approach phase cannot yet be described in simple terms. It terminates sharply, followed by a series of pulses of shortening duration and high repetition rate called the terminal phase (Fig. 2). In the terminal phase, presumably, the bat closes in on, captures, and begins to consume the insect.

The four best pursuits recognizably occupy 560, 640, 690, and 740 msec., as measured from the beginning of the first recognized increase in pulse duration. The terminal phase occupies 180 to 190 msec. of this period. The approach phase, therefore, lasts 380 to 560 msec., more or less. These measurements are objective except for the need to choose inflection points—the beginnings of the approach and terminal phases.

If one plots all of the pursuits (pulse duration *vs.* time) together by assuming that the end of the terminal phase in each case represented the same situation (capture of a fruit fly) and that the bat's flight speed was uniform and constant, then mean pulse duration at any point in time from a target can also be plotted (Fig. 3). The first discernible increase of mean pulse duration occurs at 730 msec. before capture. The beginning of the terminal phase appears to be clearly defined at 180 msec. before its end. The mean approach phase, therefore, lasts 550 msec.

A complete analysis, knowing only the time separating the flying bat from its flying target, is impossible. However, time can be converted to distance if the velocities of bat and target are known. The bat's normal flight speed was measured by timing it as it flew around the laboratory. When not occupied in pursuits, its speed was regular and deviated little from 4.5 mm./msec. Then, assuming the bat's velocity relative to the insect to be constant, one can calculate their relative positions. We know the bat's velocity is not constant. Its direction certainly changes in pursuits and its speed almost surely does, too. Nevertheless, the approximation seems useful. The fly's change in position during 750 msec. is probably small compared with the bat's (especially if the fly does not take evasive action). In any event, lacking direct observations, we must ignore the fly's motion at present. We have filmed some pursuits, but film speed and parallax problems still preclude objective quantitative statements about changes in flight speed. Thus, fixing the insect's position as zero at the end of the terminal phase, we arbitrarily calculate the bat's relative positions during each pursuit. We find that the bat in the four pursuits cited above entered the approach phase at 2.5, 2.9, 3.1, and 3.4 m. from the fruit fly, respectively, and that the terminal phase began at 0.8 to 0.9 m. The mean synchronized approach

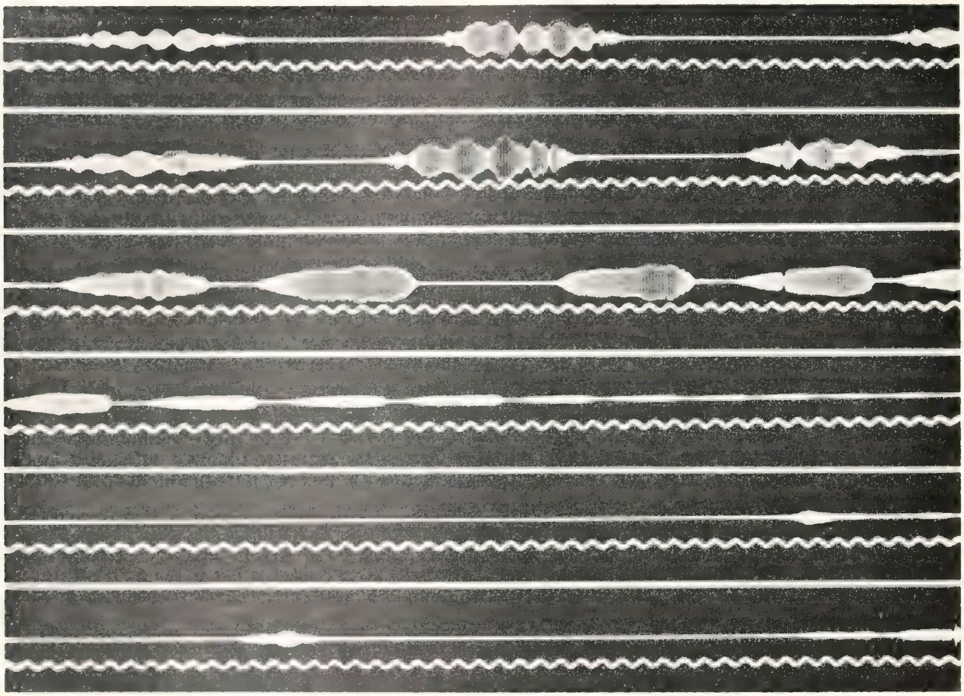


FIGURE 1. An oscillogram showing the sounds produced by *C. parnellii* in pursuit of a fruit fly. The six lines are sequential in time, reading from upper left to lower right. The end of each line is repeated at the beginning of the next line to facilitate reconstruction. The search phase preceded the portion of the pursuit shown here. The approach phase in this record starts with the first pulse shown. The terminal phase starts with the first pulse of the third line. The fourth and fifth lines show the typical relatively silent behavior that follows a capture. The frequency of the time marker is 330/sec. 1 msec. = 4.5 mm.

phase began at 3.2 m.; the terminal phase at 0.8 m. The mean approach phase, therefore, lasted 2.4 m. (Fig. 3).

Having calculated the bat's position relative to the fruit fly, one can calculate the time taken for sound to traverse the round-trip distance. One then finds that the first pulse-echo overlap (the first point at which the echo returns to the bat's ear while the pulse is still being emitted, using 350 mm./msec. as the speed of sound) occurs at 3.1, 3.3, 3.5, and 3.7 mm., respectively, or about 0.6 to 0.7 m. before the first discernible behavioral change (pulse duration increase). If the bat is aware of this first overlap and responding to it, a response time of 130–150 msec. is indicated. Two or even three successive overlaps might, of course, be required to draw attention to or confirm the presence of an interesting target. The second overlapping pulses occur at 2.8, 3.1, 3.3, and 3.5 m. *vs.* the first clearly longer pulses at 2.3, 2.6, 2.9, and 3.1 m., respectively. The third overlapping pulses are at 2.5, 2.9, 3.1, and 3.1 m., respectively.

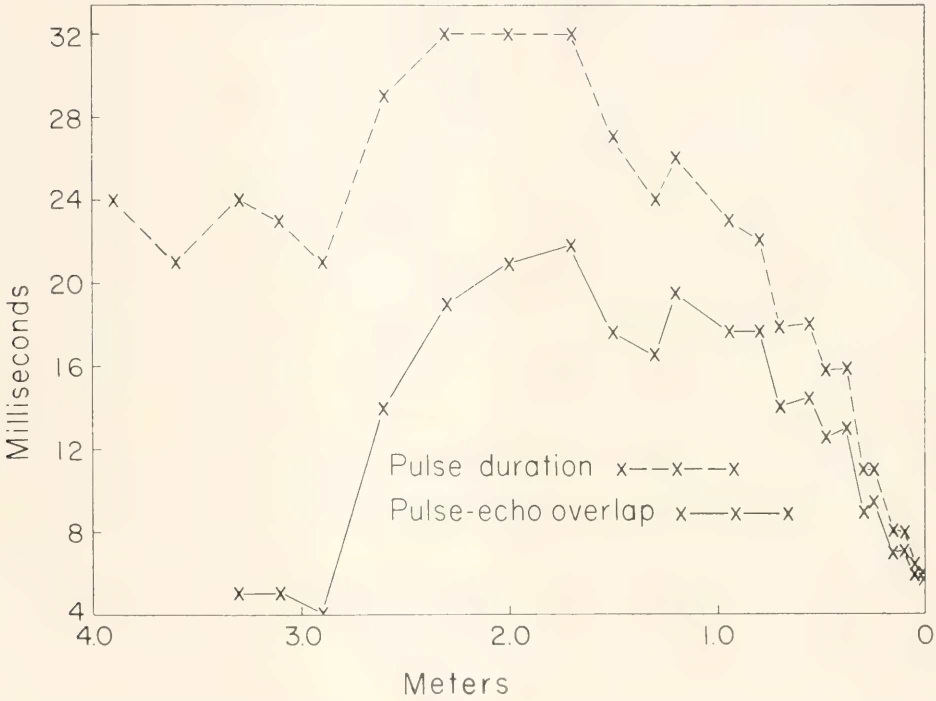


FIGURE 2. One example of a *C. parnellii* pursuit of a fruit fly. Pulse duration and calculated pulse-echo overlap are plotted against calculated distance from the insect target. Distance can be reconverted to time. 4.5 m. = 1 sec.

By 3.2 m., the synchronized mean pulse-echo overlap is 4 mesc. and the pulse-echo overlap *vs.* distance curve is a straight line from there to 1.7 m. Since the bat's speed has been assumed to be constant and the speed of sound is constant, the varying quantity contributing to the shape of this curve is the change (increase) in pulse duration. Not unlikely, the pulse duration increase is a response to the first discernible pulse-echo overlap. The bat might confirm the presence of an interesting echo with a second pulse and overlap before responding by lengthening the third pulse. The roughly regular correlation between the occurrence of overlap and the change in pulse duration also tends to delineate and confirm the usefulness of the approximations used in the calculations.

If pulses of a characteristic length are used and if overlap is required for detection, then pulse length defines the range at which detection can occur. Such a range would also require sufficient echo intensity. Thus, other things being equal, longer pulses allowing greater range might be expected to be louder and shorter ones fainter. *C. parnellii* is as loud as *Rhinolophus*, which also uses long pulses.

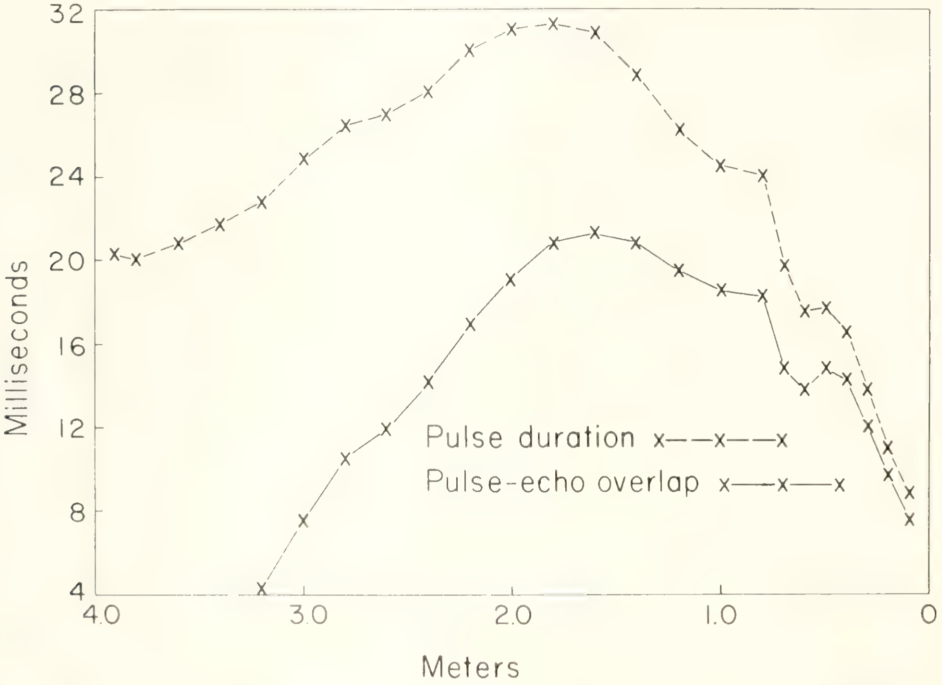


FIGURE 3. A synchronized presentation of four insect pursuits. Mean pulse duration and mean pulse-echo overlap are plotted against distance from the insect target. Distance can be reconverted to time. 4.5 m. = 1 sec.

In 17 pulses immediately preceding these four best pursuits, pulse duration ranged from 14 to 26 msec. (mean and median 22 msec.; 14 between 19 and 25 msec.). This would give pulse-echo overlap only when the bat was within about 3.8 m. of the target. If 2 msec. of overlap were required for initial analysis, then about 3.5 m. would be the maximum range.

The mean synchronized pulse duration starts upward from 22 msec. at 3.3 m., reaching a maximum of 31 msec. at 2.0 m. (Fig. 3). At this point the pulse-echo overlap is 19 msec. The mean pulse duration holds steady to 1.6 m.

and then drops just enough down to 0.8 m. to hold the pulse-echo overlap between 19 and 21 msec. Indeed, the overlap range of 18–21 msec. includes the distance between 2.1 and 0.8 m., with mean pulse duration first rising from 30 to 31 and then dropping to 24 msec. The approach phase might be subdivided then into the portion from 3.2 m. to 2.1 m., during which pulse duration rises uniformly from 22 to 30 msec. and pulse-echo overlap rises from 4 to 18 msec., and a second subphase, where pulse duration lengthens slightly and then shortens to hold the mean pulse-echo overlap between 18 and 21 msec. During this time, the range of raw pulse-echo overlaps is actually 11 to 25 msec. but 17 of 26 are between 17 and 22 msec. Probably some advantage accrues from a pulse-echo overlap of about 17 to 22 msec. Presumably some measurement or comparison occurs during this time.

The pulse duration in individual pursuits rose and fell as follows during the approach phase: (1) from 21 msec. initially to 32 msec. at 1.7 m. to 23 msec. at 0.8 m.; (2) from 20 msec. initially to 37 msec. at 2.0 m. to 26 msec. at 0.85 m.; (3) from 21 msec. initially to 33 msec. at 1.4 m. to 27 msec. at 0.8 m.; (4) from 20 msec. initially to 28 msec. at 1.65 m. to 23 msec. at 0.8 m.

The approach phase includes 8, 9, 10, and 7 pulses, respectively. Four, 6, 7, and 5 pulses were required, respectively, from the first calculated overlap to the point at which the characteristic overlap range of 17–22 msec. was achieved.

During the approach phase, the interpulse interval shortens somewhat. The pulses tend to occur in pairs—a short interpulse interval alternating with a long. The longer intervals seem to shorten while the shorter intervals remain more constant.

In one example, the search phase interpulse intervals (expressed as the silent period from the end of one pulse to the onset of the next) range from 21 to 57 msec. in the following sequence—29, 57, 29, 50, 24, 47, 21, 26, 53. In the approach phase, there is a tendency to shorten. The subsequent pairs in the same example are 29, 46, 25, 42, 21, and 36 msec. In the late approach phase or transition to terminal phase, the sequence is 4, 4, 19, 5, and 20 msec. In the terminal phase, interpulse interval is uniformly about 2 to 5 msec. We are tempted to speculate that the pairing of pulses might be useful for directional localization, the bat comparing closely spaced echoes with his head or ears slightly changed in position.

The terminal phase—between 0.8 and 0 m. or between 180 and 0 msec.—is characterized not only by minimal interpulse intervals but by the drastic and regular shortening of pulses. Thus, the mean synchronized pulse duration drops from 24 msec. at 0.8 to 16.5 msec. at 0.4 m. and 9 msec. at 0.1 m. Pulse duration drops 1 msec./4.9 cm. Individually, the terminal pulses went down literally as well, but in paired steps. Thus, one sequence was 22, 18, 18, 15.5, 15.5, 11, 11, 8, 8, 6, 5.8 msec., respectively. Pairing may mean that two pulses may follow every positive decision. Perhaps they are compared to yield information on closing speed.

In individual pursuits, the slope of pulse duration *vs.* distance from target of the terminal phase varied from about 1 msec./2.5 cm. to 1 msec./7.5 cm. Choosing the slope is somewhat arbitrary. The very last pulse of the four series varies from 5.8 to 8.2 msec. in duration.

The overlap between pulse and echo in the terminal phase drops linearly, essentially parallel with pulse duration. The mean pulse duration shortens and the bat continues to approach the target so that overlap occupies an increasing proportion of each pulse, but a decreasing absolute value. The mean synchronized overlap drops from 18 msec. at 0.8 m. to 12 msec. at 0.3 m. and 7.5 msec. at 0.1 m. In a specific example, the sequence of pulse-echo overlaps is 17, 14, 14.5, 12.5, 13, 9, 9.5, 7, 7, 6, 5.5 msec., respectively.

In *C. parnellii,* therefore, we see a characteristic increase in pulse duration associated temporally apparently with detection of an insect prey by pulse-echo overlap. This fixes the maximum range at about 3.8 m. Recognized behavioral changes occur at an average distance of 3.3 m. Thereafter pulses continue to lengthen until the pulse-echo overlap exceeds 17 or 18 msec. Such overlap, averaging 18 to 21 msec., characterizes the approach from 2.1 to 0.8 m. Thereafter, pulse duration is drastically shortened in a sequential and essentially linear fashion to about 9 msec. at 0.1 m. with a parallel decrease in overlap to about 7.5 msec. at 0.1 m. Interpulse intervals range between about 20 and 60 msec. during the approach phase but rapidly shorten to about 2.5 msec. in the terminal phase.

C. parnellii appears to detect insects by pulse-echo overlap. The last part of each pulse, about 2 msec., is frequency modulated. This may facilitate the detection of a faint returning echo (from a fruit fly at over 3 m., for example) by ensuring a frequency difference between the outgoing pulse and the echo. With the bat flying at 4.5 mm./msec., it traverses some 100 mm. during one pulse and another 100 to 300 mm. during one interval, making 200 to 400 mm. during the total and reducing the round-trip distance from the fly by 400 to 800 mm. With sound travelling at 35 mm./msec., if the pulse duration were kept constant, then an echo which just missed overlapping one pulse would overlap the next by about 1 to 2 msec. The interpulse interval, therefore, may be a consequence of the bat's flight speed—designed to survey all echoes by overlapping them initially with the terminal pulse frequency drop.

The searching pulse duration may also be a consequence of the range at which a faint echo can be detected physically. Once having detected the echo, the pulses can be lengthened since echo existence is now established and the range can be presumed to be decreasing. A longer pulse would overlap echoes from a greater range but these might be too attenuated to be audible. Interestingly, bats with unusually long pulses, perhaps designed for long range detection (*Rhinolophus* and *C. parnellii*), produce high intensities.

Presumably a second or third short pulse-echo overlap is either desirable or unavoidable while the bat either recognizes or examines the echo. Then a rapid switch to overlaps of about 20 msec. occurs. This now removes the pulse-echo overlap from the frequency modulated part of the pulse to the constant frequency portion. Either some comparison is required between the pulse and echo or else the bat requires 20 msec. in order to assess some echo quality.

Let us consider now only overlap with the constant frequency portion of the pulse. The bat hears an attenuated outgoing pulse (Henson, 1964) and a reflection. At least two useful mechanisms can be visualized.

First, the bat may use the outgoing sound as a reference for the returning echo in a linear detector. It would achieve an improvement in signal: noise ratio because it could compare the returning echo with a reference frequency. Such a system would require closely controlled frequency constancy or the beginning of echo detection would be uncertain by a number of wave-lengths. In addition, relative movement of bat and target introduces complications—chiefly Doppler shift. Doppler shift could, indeed, be the quantity assessed in these 20 msec., giving a measure of the relative speed of the target. What the effect or role of the slight frequency rise at the beginning of the pulse and, therefore, at the beginning of the echo would be cannot be formulated now. Nor can we yet extensively interpret the role of the terminal frequency drop.

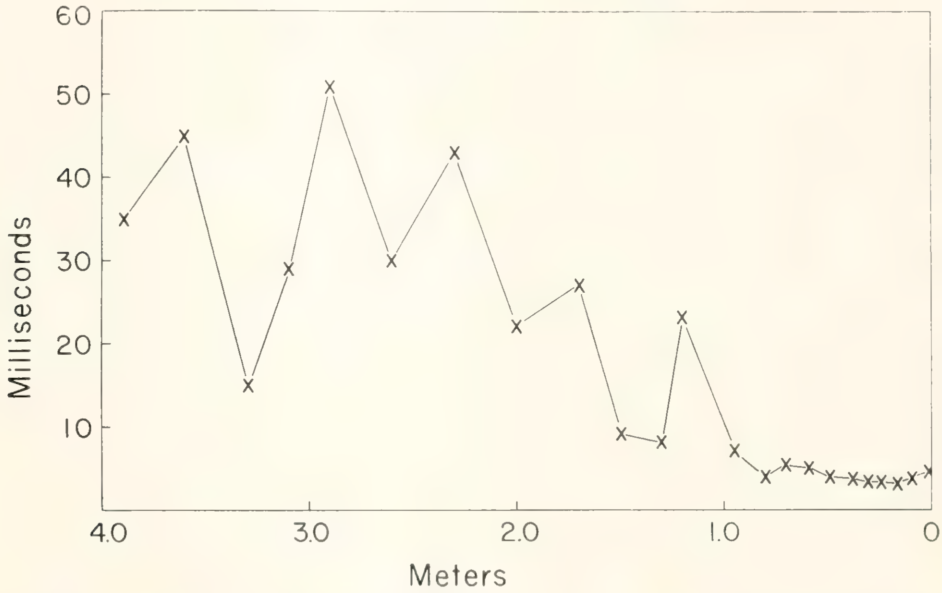


FIGURE 4. One example of a *C. parnellii* pursuit of a fruit fly. The duration of the silent period between pulses is plotted against the calculated distance from the target at the beginning of the pulse preceding each interval. Distance can be reconverted to time. 4.5 m. = 1 sec.

Second, the pulse and the echo could be heterodyned in the ear (Wever and Lawrence, 1954). Since there is relative motion between bat and insect the echo is Doppler-shifted and the synthesized signal contains sum and difference frequency components in addition to the pulse and echo frequencies. The difference frequency may be viewed as a relatively slow amplitude modulation of a basic signal: with a closing speed of 5 mm./msec. and an output pulse frequency of 34 kc this modulation frequency would be about 1 kc. The onset of the modulations would give the target distance (by elapsed time) and the frequency of these modulations would give the closing speed. This method should be useful if the ratio of perceived outgoing signal/reflection were between 0.1 and 10, but at present we are not prepared to evaluate the situation quantitatively. It may be noted that in principle the bat could make similar use of the sum frequency as well, since its presumed frequency perception extends to the 70 kc region (Wever and

Vernon, 1961; Henson, 1964). The presence of frequency rise at the beginning of the pulse and frequency drop at the end does not change the nature of the conclusions: for example, the overlap of the end of the pulse (with decreasing frequency) with an echo (at constant frequency) would introduce frequency modulation into the amplitude fluctuations at the difference frequency as well as increase the frequency of the amplitude modulations. Some aspects of pulse-echo overlap have been discussed previously by Pye (1960, 1961a, 1961b) and Kay (1961, 1962).

Only at 0.8 m. does the bat abandon such long overlap. Thereafter what governs pulse duration and overlap? At these distances overlap almost equals pulse duration. The short interpulse intervals here are long enough apparently to preclude overlap between an echo of one pulse and the subsequent pulse. That is, the bat receives the echo fully before it starts to emit the next pulse. The close timing here is interesting but lends itself to date only to speculative interpretation. Clearly, we are limited here by reaction time in the central nervous system. The timing may reflect the length of the path involved in the decisions in this part of the pursuit. Henson (1964) has shown that in *Tadarida* the stapedius muscle contracts about 7 msec. before the bat emits a pulse. This implies that the decision to make a pulse has been made at least 7 msec. before its emission. Novick and Griffin (1961) similarly found that the cricothyroid muscle of the larynx showed trains of muscle action potentials some 4 to 20 msec. before the emission of a pulse. Presumably such factors put limits on pulse repetition rate and the design of the terminal phase.

The average pulse duration almost parallels the average pulse-echo overlap in the terminal phase because it is shortened roughly proportionally to bat-insect distance. Such is not the case for the individual pursuits. It may well be that the differences represent the target's flight speed relative to the bat. With so few sequences this cannot be assessed.

The closer and closer approach of echo overlap to pulse initiation could well be a clue to range. In the terminal phase, presumably more frequent assessment is desirable—short pulses and short interpulse intervals. The limiting factors are obscure.

In *Pteronotus*, the first behavioral indication of an insect pursuit (as in the vespertilionids) is a shortening pulse-to-pulse interval. The pulse duration, normally about 4 msec., is apparently set during the approach phase so as to yield a pulse-echo overlap of about 1.5 msec. The difference between 1.5 msec. and 20 msec. of overlap is especially interesting since the targets were the same (*Drosophila*) and the bats are close relatives (congeneric according to Burt and Stirton, 1961). In *Pteronotus* (Novick, 1963b), apparently purposeful pulse-lengthening sometimes immediately follows the first pulse-echo overlap. In *C. parnellii*, such lengthening is regular. Thus, the presumably required pulse-echo overlap is achieved more rapidly than would be produced simply by the bat's pursuing the fly. *Pteronotus* was apparently restricted to a range of about 700 mm. by its pulse duration of about 4 msec. Its pulse intensity is much less than that of *C. parnellii*.

In *Pteronotus*, terminal phase pulse durations of 2 to 1 msec. permit a fixed pulse-echo overlap of about 1.1 msec. In *C. parnellii*, such short pulses may be physically impossible or the exact relationship may differ. In any event, pulse-echo overlap is not kept constant but decreases rapidly.

In *Pteronotus*, the pulse-to-pulse interval is short and decreases in a regular pattern (about 50 to 25 msec.) during the approach phase and is then minimal and uniform (about 7 to 4.5 msec.) in the terminal phase. This minimal terminal pulse-to-pulse interval is shared by *C. parnellii*. The range of 50 to 25 msec. in the approach phase is interesting since in *C. parnellii* this range also occurs but not in the same regular sequence. Approach phase decisions (presumably because of conduction time and synaptic delays) must occupy 25 to 50 msec., perhaps some types of decisions depending on one interval, others on the other.

SUMMARY

1. Insect pursuits by *C. parnellii* can be divided into search, approach, and terminal phases.

2. *C. parnellii,* using pulses of about 21 msec. in duration, appears to detect insects initially by pulse-echo overlap. This sets the maximum range of detection at about 3.8 m. and presumably requires the use of high intensity pulses.

3. Recognized behavioral changes (increased pulse duration) occur at about 3.3 m.

4. Following detection, pulse duration increases until pulse-echo overlap exceeds 17–18 msec.

5. Pulse-echo overlaps of 18–21 msec. characterize the pursuit from 2.1 to 0.8 m., after which shortening of pulse duration and closing on the insect sharply reduce pulse-echo overlap to about 7.5 msec. at 0.1 m.

6. Interpulse intervals range from about 20 to 60 msec. during the approach phase but shorten abruptly to about 2.5 msec. in the terminal phase.

7. Several speculative interpretations of the factors controlling pulse repetition rate are discussed. Speculative views of the uses of pulse-echo overlaps of 20 msec. are also discussed.

LITERATURE CITED

Burt, W. H., and R. A. Stirton, 1961. The Mammals of El Salvador. *Misc. Publ.*, Mus. Zool., Univ. Mich. No. 117: 1–69.

Cahlander, D. A., J. J. G. McCue and F. A. Webster, 1963. The determination of distance by echolocating bats. *Amer. Zool.*, **3**: 527.

Griffin, D. R., 1953. Bat sounds under natural conditions, with evidence for echolocation of insect prey. *J. Exp. Zool.*, **123**: 435–466.

Griffin, D. R., 1958. Listening in the Dark. Yale University Press, New Haven, Conn.

Griffin, D. D., 1962. Comparative studies of the orientation sounds of bats. *Symp. Zool. Soc. Lond.*, **7**: 61–72.

Griffin, D. R., and A. Novick, 1955. Acoustic orientation in neotropical bats. *J. Exp. Zool.*, **130**: 251–300.

Griffin, D. R., F. A. Webster and C. R. Michael, 1960. The echolocation of flying insects by bats. *Animal Behav.*, **8**: 141–151.

Grinnell, A., and D. R. Griffin, 1958. The sensitivity of echolocation in bats. *Biol. Bull.*, **114**: 10–22.

Hall, E. R., and K. R. Nelson, 1959. The Mammals of North America. Ronald Press Co., New York, N. Y.

Henson, O'D. W., Jr., 1964. Echolocation and hearing in bats with special reference to the function of the middle ear muscles. A thesis deposited in the library of Yale University, New Haven, Conn.

Kay, L., 1961. Perception of distance in animal echolocation. *Nature,* **190**: 361.

Kay, L., 1962. A plausible explanation of the bat's echo-location acuity. *Animal Behav.,* **10**: 34–41.

Novick, A., 1958. Orientation in paleotropical bats. I. Microchiroptera. *J. Exp. Zool.,* **138**: 81–154.

Novick, A., 1962. Orientation in neotropical bats. I. Natalidae and Emballonuridae. *J. Mammal.,* **43**: 449–455.

Novick, A., 1963a. Orientation in neotropical bats. II. Phyllostomatidae and Desmodontidae. *J. Mammal.,* **44**: 44–56.

Novick, A., 1963b. Pulse duration in the echolocation of insects by the bat, *Pteronotus. Ergebnisse Biol.,* **26**: 21–26.

Novick, A., and D. R. Griffin, 1961. Laryngeal mechanisms in bats for the production of orientation sounds. *J. Exp. Zool.,* **148**: 125–145.

Pye, D., 1960. A theory of echolocation by bats. *J. Laryn. Otol.,* **74**: 718–729.

Pye, D., 1961a. Perception of distance in animal echolocation. *Nature,* **190**: 362–363.

Pye, D., 1961b. Echolocation by bats. *Endeavour,* **20**: 101–111.

Wever, E. G., and M. Lawrence, 1954. Physiological Acoustics. Princeton Univ. Press., Princeton, New Jersey.

Wever, E. G., and J. A. Vernon, 1961. Hearing in the bat, *Myotis lucifugus,* as shown by the cochlear potentials. *J. Auditory Res.,* **2**: 158–175.

THE TEMPERATURE-COEFFICIENTS OF RIBONUCLEASES FROM TWO SPECIES OF GASTROPOD MOLLUSCS FROM DIFFERENT THERMAL ENVIRONMENTS

KENNETH R. H. READ[1]

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Little is known about adaptive differences between temperature-coefficients or activation energies of the enzymes of animals from different thermal environments. This paper supplements our meager knowledge in this field.

The most recent work concerning adaptation at the level of activation energies includes that of Vroman and Brown (1963), Mutchmor and Richards (1961), Lin and Richards (1956) and Kenney and Richards (1955). Richards and co-workers have concerned themselves with arthropod apyrase; they have questioned much of the earlier work on this enzyme on the basis of chemical reasoning (Mutchmor and Richards, 1961). Mutchmor and Richards (1961) have shown that the lower the chill-coma temperature of an arthropod, the higher the activation energy of its muscle apyrase; this may be adaptive since, for arthropods that normally live close to their chill-coma temperatures, an increase in temperature only slightly above that of chill-coma may result in apyrase activity in excess of the minimum required to support muscular function; forms living well above chill-coma temperatures have little need of such an adaptation. On the other hand, Lin and Richards (1956) failed to detect any differences between the activation energies of "proteinase" and invertase from two species of insects with different chill-coma temperatures. The work of Vroman and Brown (1963) on rat and frog liver succinic dehydrogenase contrasts with that of Richards and co-workers but, as suggested by Read (1964), Vroman and Brown neglected their data for the lowest temperature tested in their frog preparation; these authors might otherwise have been led to a conclusion similar to that of Richards and co-workers for apyrase.

In this work the activation energies of ribonuclease components isolated by chromatography from the digestive glands of the gastropods, *Buccinum undatum* L., a form limited to boreal seas, and *Fasciolaria tulipa* L., a species limited to warm temperate to tropical seas, are studied. The two species have similar modes of life, both being scavenging carnivores; they belong to the same super-family, *i.e.*, Buccinacea. The report also provides data concerning molluscan ribonuclease which has previously been studied in squid by Roth and co-workers (Roth and Bachmurski, 1957; Roth, 1959; Edmunds and Roth, 1960).

MATERIALS AND METHODS

General

Buccinum undatum L. was collected in June in the Salt Pond, Blue Hill, Maine, and in Eggemoggin Reach, off High Head, Brooklin, Maine. *Fasciolaria tulipa* L. was collected from Bimini Lagoon, Bimini, Bahama Islands, in April.

---

[1] Present address: Department of Biology, Boston University, Boston, Mass. 02215.

After the animals had been collected their shells were cracked and the digestive glands pulled free of the remainder of the bodies. The digestive glands were dissected from as much gonad and alimentary tract tissue as possible prior to storage at −20° C.; the possibility that the digestive gland enzymes were contaminated by those from other tissues has to be entertained. Stomachs were empty at the time of dissection.

Material obtained in Bimini was transported packed in dry ice; that from Maine was frozen into a large block of ice which was well insulated for the journey to Boston; all material was frozen on arrival.

Assay for ribonuclease activity

Ribonuclease activity was assayed by a modification of the method of Anfinsen *et al.* (1954). Except where otherwise noted, the procedure used was the following: 1 ml. of pH 5, 0.67 ionic strength acetate buffer and 0.5 ml. of enzyme solution were placed in a test tube; at zero time 1.0 ml. of a 0.4% or 1% solution of ribonucleic acid in water was added; the tube contents were then immediately mixed and the tubes placed in a thermostat for periods ranging from 25 minutes for an incubation temperature of 37° C. to 5 hours for an incubation temperature of 5° C. At the end of this time 0.5 ml. of 0.75% uranyl acetate in 25% perchloric acid was added and the tube contents again mixed; the resulting precipitate was removed by centrifugation. A 0.1-ml. aliquot of the supernatant was then diluted to 3.1 ml. with distilled water and its absorbancy read at 260 mμ. Reagent blanks were run concurrently with the samples undergoing assay. The unit of enzyme activity is defined as an increase, $\Delta(\text{O.D.})_{260}$, of one optical density unit over the reagent blank under the standard conditions of the assay; enzyme blanks, incubated without substrate, were also run.

Purification of substrate ribonucleic acid

Commercial grade yeast ribonucleic acid was dissolved in 1 *M* sodium acetate and dialyzed for three days against frequent changes of distilled water. At the end of this time the dialyzed solution was lyophilized. The resulting material gave a reagent blank at 37° C. in pH 5, 0.27 ionic strength, acetate buffer of about 0.065 optical density unit when read against distilled water.

Purification of ribonucleases

The procedure described below was essentially that followed for the purification of the ribonucleases. All operations were carried out in a cold room at 0–4° C.

Approximately 100 g. of tissue were homogenized with three volumes of distilled water in a Waring Blendor at 4° C. The homogenate was brought to about pH 4 by the addition of glacial acetic acid and centrifuged at 15,000 *g* for 10 minutes. The clarified solution at pH 4 was saturated with ammonium sulfate (75 g./100 ml.), allowed to stand for approximately an hour and centrifuged. The supernatant was discarded and the residue resuspended in distilled water; this suspension was dialyzed for about 20 hours against pH 5, 0.067 ionic strength, acetate buffer. The dialyzed solution was assayed over a wide range of pH before

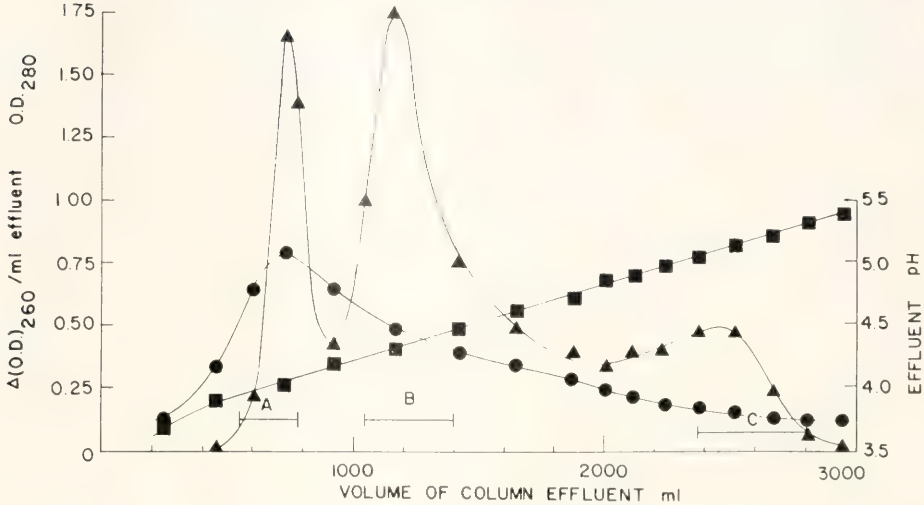


FIGURE 1. Second chromatography of *Buccinum undatum* preparation from 77 g. tissue. Ribonuclease activity of column eluate at pH 5 ▲; OD_{280} of eluate ●; pH of eluate at 5° C. ■. Varigrade elution system: 1 l. each of the following citric acid-sodium citrate buffers: 0.2 *M*, pH 3.9; 0.2 *M*, pH 5.0; 0.2 *M*, pH 6.0; 0.5 *M* pure sodium citrate. Assay temperature: 37° C.

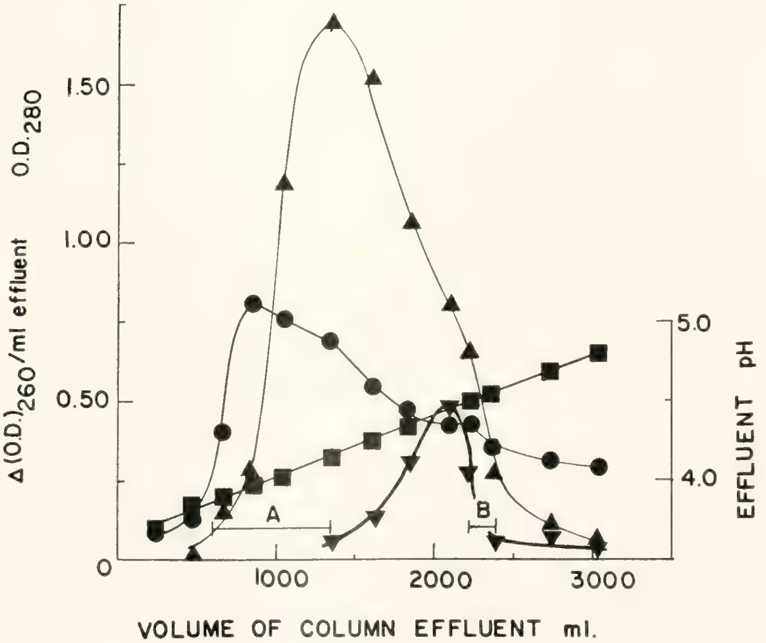


FIGURE 2. Second chromatography of *Fasciolaria tulipa* preparation from 102 g. tissue. Symbols as for Figure 1; ribonuclease activity at pH 7.25 ▼. Varigrade elution system: 1 l. each of the following citric acid-sodium citrate buffers: 0.2 *M*, pH 3.8; 0.2 *M*, pH 4.6; 0.2 *M*, pH 5.2; 0.2 *M*, pH 6.0. Assay temperature: 37° C.

proceeding with the next step involving chromatography on phosphocellulose. To effect this, the dialyzed solution was brought to a pH ranging between 3.2 and 4.0 and filtered through washed phosphocellulose adjusted to the same pH. The column was then washed with a few liters of 0.05 *M* (in citric acid-sodium citrate

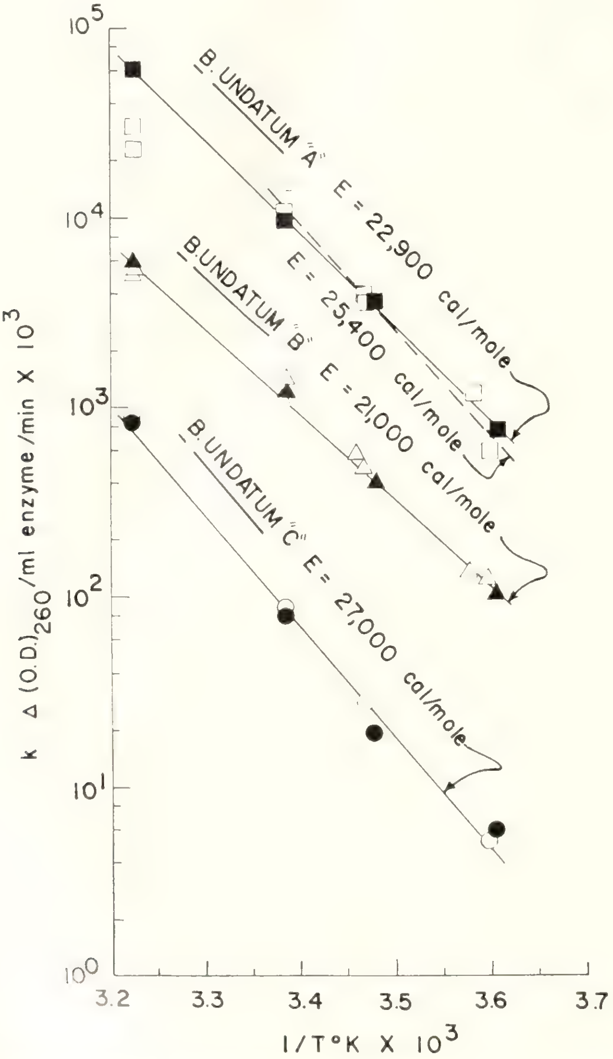


FIGURE 5. Arrhenius plot of activity of ribonuclease A, B and C components of *Buccinum undatum.* Component A ■ □; component B ▲ △; component C ● ○. Closed symbols indicate 1% ribonucleic acid; open symbols 0.4% ribonucleic acid. Divide ordinate by 10 for activity of B component, by 100 for that of A component. Activation energy of 22,900 cal./mole computed from data at 1% substrate concentration over temperature range 5–37° C. (1/T × 10³ = 3.6 – 3.2). Activation energy of 25,400 cal./mole computed from data at 0.4% and 1% substrate concentration over temperature range 5–22.5° C. (1/T × 10³ = 3.6 – 3.4).

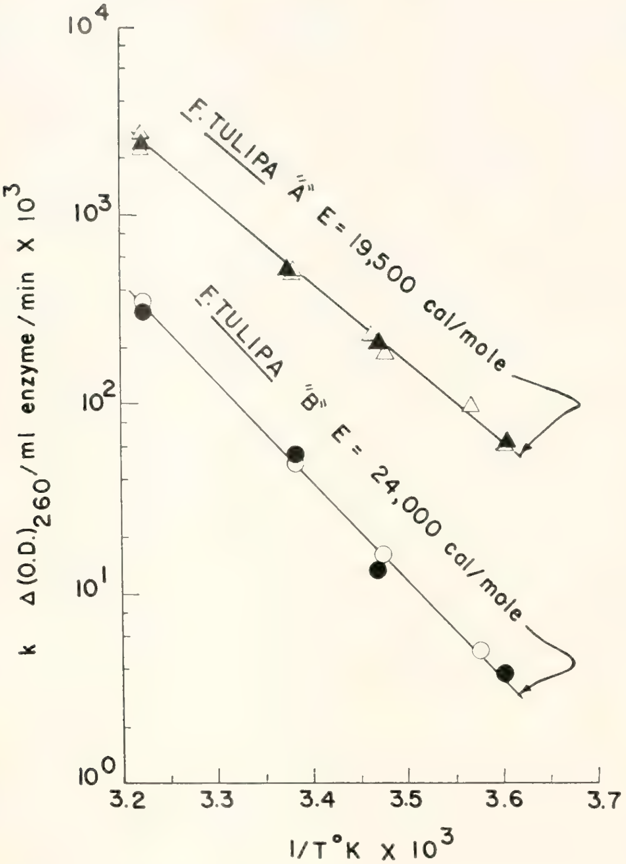


FIGURE 4. Arrhenius plot of activity of ribonuclease A and B components of *Fasciolaria tulipa;* component A ▲ △ ; component B ● ○ ; closed and open symbols as described for Figure 3. Divide ordinate by 10 for activity of A component.

combined) citrate buffer of pH 3.2–4.0. After being washed the column was eluted using a four-compartment varigrade apparatus and 0.2 *M* citrate buffers ranging in pH from 3.8 to that of pure sodium citrate. Most of the ribonuclease activity was recovered in the effluent.

After elution the resulting fractions containing ribonuclease activity were pooled, lyophilized, redissolved in distilled water, dialyzed against 0.067 ionic strength, pH 5, acetate buffer and rechromatographed on phosphocellulose. The resulting fractions were dialyzed against 0.2 *M* ammonium bicarbonate, lyophilized and stored at −20° C.

RESULTS

Figures 1 and 2 indicate the results of the chromatography of the digestive gland preparations. *Buccinum undatum* gives rise to three components of ribonuclease activity, *Fasciolaria tulipa* to two. All components exhibited maximal activity in acetate buffer in the neighborhood of pH 5. A preparation from *Busycon canaliculatum* was also tested and gave an optimum at about pH 5; the drop-off in activity on either side of the optimum was not due to irreversible inactivation since the preparation was stable at 37° C. in pH 3 and pH 8 buffers for an interval equal to that of the incubation period. On the other hand, preparations from *Livona pica* L., *Strombus gigas* L. and *Cassis tuberosa* L. gave optima at about pH 4; *Cassis tuberosa* also gave an additional optimum at about pH 7.

The *Fasciolaria tulipa* preparation gave rise to two non-superposable major peaks when ribonuclease activity was assayed at pH 5 in acetate and 7.25 in trishydroxymethylaminomethane-HCl buffers; in Figure 2 the bump in the pH 5 curve coincides with the peak at pH 7.25. This indicates that the pH 5 peak is composed of at least two different ribonuclease components.

Enzyme kinetics

Arrhenius plots for the several *Buccinum undatum* and *Fasciolaria tulipa* components of ribonuclease activity at pH 5 in 0.27 ionic strength acetate buffer are shown in Figures 3 and 4. Activation energies associated with the *Buccinum undatum* A, B and C components are 22,900 or 25,400 (depending on the data

TABLE I

Statistical limits of confidence in differences between activation energies associated with various components

	Buccinum undatum A*	*Buccinum undatum* A†	*Buccinum undatum* B	*Buccinum undatum* C	*Fasciolaria tulipa* A	*Fasciolaria tulipa* B
Buccinum undatum A*						
Buccinum undatum A†	$0.90 < P < 0.95$					
Buccinum undatum B		—	—			
Buccinum undatum C		—	—			
Fasciolaria tulipa A	$0.999 < P$	$0.999 < P$	$0.98 < P < 0.99$			
Fasciolaria tulipa B	—	—	—	$0.99 < P < 0.999$		

* Computed from data obtained with 1% substrate concentration over the temperature range 5–37° C. See Figure 3.

† Computed from data obtained with 0.4% and 1% substrate concentration over the temperature range 5–22.5° C. See Figure 3.

used), 21,000 and 27,000 cal./mole, respectively; for *Fasciolaria tulipa* the corresponding values are 19,500 for the A component and 24,000 cal./mole for the B component.

Values for reaction rates, *k,* were obtained by extrapolating plots of the extent of reaction *vs.* concentration of enzyme to zero enzyme concentration; the tangents at the origin divided by the times of incubation, which ranged from 25 minutes at 37° C. to 5 hours at 5° C., were taken as the reaction velocities for use in the Arrhenius plots. The *Buccinum undatum* A component gave rise to pronounced downward curvature in plots of enzyme activity *vs.* enzyme concentration at 22.5° C. and 37° C. at 0.4% substrate concentration; an accurate determination of the tangent was thus difficult; at 1% substrate concentration the downward curvature was less apparent and the tangent at the origin could be estimated with greater precision. The other preparations gave more or less linear plots, especially at the lower enzyme concentrations.

When dissolved in buffer all preparations were stable through cycles of freezing, thawing and warming to room temperature, as indicated by the close grouping of the points at each temperature in Figures 3 and 4. These points represent determinations conducted on different days with the same alternately thawed and frozen solution of enzyme. All preparations were stable at 37° C. and pH 5 for 25 minutes.

Slopes of the lines in the Arrhenius plots were computed from the data by the method of least squares and statistical limits of confidence for their differences are given in Table I. The *Buccinum undatum* components as a group have slightly higher activation energies than those of *Fasciolaria tulipa.*

Discussion

Chromatography

The interpretation of the results of the ion-exchange chromatography of proteins should be approached with caution. The presence of multiple peaks of a given type of enzymatic activity may merely indicate that the enzyme in question has given rise to a number of preparative artifacts; evidence for these has recently been treated in a review by Kaplan (1963).

pH optimum

All preparations studied had pH optima in the neighborhood of pH 4–5; these are somewhat lower than the pH optima of mammalian acid ribonucleases. For the bovine pancreatic enzyme Kalnitsky *et al.* (1959) found an optimum at about pH 6.2 in 0.43 ionic strength buffer. De Lamirande and Allard (1959) obtained optima at pH 5.8 for rat liver enzyme and pH 5.5 for rat brain enzyme. Maver *et al.* (1959) found similar values for calf spleen ribonuclease. For heated squid caecal fluid enzyme, Edmonds and Roth (1960) demonstrated activity through the range pH 4–7 with a peak at about pH 5.2. Similar results were obtained for a squid gill preparation.

Enzyme kinetics

The determination of the activation energies of the complex reactions involved in this work is fraught with uncertainties. The first of these involves changes in the Michaelis constant, K_m, of the enzymes with temperature; if these are not taken into account, erroneous values of V may result (Dixon and Webb, 1958). This complication can be avoided by determining the activation energies associated with each enzyme at two different substrate concentrations; if identical Arrhenius plots are so obtained, the enzymes can be considered saturated with substrate at each temperature. The second complication is inherent in the complexity of the substrate molecule. Thus, the enzyme reacts with the original substrate molecule producing fragments, probably of variable composition, that decrease in size with time. These may be subsequently attacked by the enzyme at different rates so that the most labile are broken down first, resulting in the enrichment of the reaction mixture with nucleotide moieties less susceptible to attack and possibly inhibiting. In view of these complications it would again seem reasonable that they could be disregarded if the reaction velocities could be proven to be independent of substrate concentration.

Accordingly, the enzyme components were assayed at two substrate concentrations, 0.4% and 1%, to clarify the possible effects of the variables just discussed. Except for the *Buccinum undatum* A component, the rate of reaction appears not to be dependent on substrate concentration and even this exception could possibly be ascribed to chance alone, as judged by the scatter of the data in Figure 3.

Values of activation energies associated with the *Buccinum undatum* A and B components and the *Fasciolaria tulipa* A component only approximate that obtained by Kalnitsky *et al.* (1959) for bovine pancreatic ribonuclease between 30 and 50° C., *i.e.*, 20,290 cal./mole.

The chromatograms for the *Buccinum undatum* and *Fasciolaria tulipa* preparations in Figures 1 and 2, respectively, indicate that the *Buccinum undatum* A, B and C, and *Fasciolaria tulipa* A components are relatively "pure" with respect to contamination with other active components; however, the possibility must be entertained that the *Fasciolaria tulipa* B component is contaminated by the *Fasciolaria tulipa* A component. This would result in a concomitant lowering of the activation energy of the *Fasciolaria tulipa* B preparation.

Ecology and comparative physiology of the species investigated

Buccinum undatum ranges from Labrador to New Jersey (Johnson, 1934) and is adapted to a cool environment. Gowanloch (1927) has exposed individuals of this species to increases of temperature of 1° C. per 5-minute interval and noted that under these conditions death occurs at 29° C.; this suggests that in nature the species would be able to survive only at temperatures well below this value.

Fasciolaria tulipa is a warm-water species and ranges from North Carolina to Florida, Texas (Johnson, 1934) and the West Indies (Warmke and Abbott, 1961). At Bimini it is found half submerged, crawling about over the exposed flats in the lagoon at low tide. During the summer the water temperature of the tide pools on the flats probably rises into the mid-thirties since surface water temperatures of the Gulf Stream near Bimini reach almost 29° C. at this time (Fuglister, 1947). *Fasciolaria tulipa* thus normally lives in a temperature range well above that lethal to *Buccinum undatum;* the enzymes of the two species might therefore be expected to be adapted accordingly.

Conclusions

The activation energies of the *Buccinum undatum* components as a group are slightly higher than those of *Fasciolaria tulipa.* Their range of variation does not approach that found by Mutchmor and Richards (1961) for arthropod apyrase; here activation energies ranging from 11,600 to 25,600 cal./mole were found and these data fit in well with the rationale of adaptation proposed by the authors. It is therefore concluded that in contrast to the apyrase of arthropods or vertebrate succinic dehydrogenase (Read, 1964), adaptation of gastropod digestive gland ribonuclease by way of variation of activation energy is either nugatory or, like that of the insect digestive enzymes described by Lin and Richards (1956), non-existent.

I thank Drs. J. T. Edsall, C. B. Anfinsen and G. Guidotti for their kind cooperation, help or suggestions during the course of this work. I am indebted to Mr. and Mrs. Peter Helburn for the loan of their cottage in Maine and to Mr. and Mrs. Winslow H. Duke, Mrs. Charles G. Loring and Mr. and Mrs. Alan T. Bemis for their help in the collection of *Buccinum undatum.* Appreciation is expressed to the American Museum of Natural History for the grant of a Lerner Marine Fellowship which allowed me the use of the Lerner Marine Laboratory of the American Museum of Natural History, Bimini, Bahama Islands.

This work was performed while the author was Paul Dudley White Fellow in Cardiology of the Massachusetts Heart Association, Inc., Plymouth County Chapter.

NOTE ADDED IN PROOF

Since this article went to press Licht (*Comp. Biochem. Physiol.,* **13**: 27–34, 1964; *ibid,* **12**: 331–340, 1964) has reported data relative to muscular contraction and temperature coefficients of alkaline phosphatase and ATPase in reptiles from different thermal environments; in addition, Baslow and Nigrelli (*Zoologica N. Y.,* **49**, 41–51, 1964) have suggested that there are differences in Q_{10} as a function of temperature in the brain cholinesterase of cold- and warm-water fish.

SUMMARY

1. *Buccinum undatum* digestive gland ribonuclease chromatographed on phosphocellulose consists of three enzymatic components, all showing an optimum pH of about pH 5 in acetate buffer. Activation energies of the three components are 21,000, 22,900 and 27,000 cal./mole at pH 5.

2. *Fasciolaria tulipa* digestive gland ribonuclease chromatographed on phosphocellulose consists of two enzymatic components, both with pH optima near pH 5 in acetate buffer. Activation energies of the two components are 19,500 and 24,000 cal./mole at pH 5.

3. It is concluded that the ribonucleases of gastropod digestive glands show little or no adaptation, by way of differences in activation energies, to the thermal environment.

LITERATURE CITED

ANFINSEN, C. B., R. R. REDFIELD, W. L. CHOATE, J. PAGE AND W. R. CARROLL, 1954. Studies on the gross structure, cross-linkages, and terminal sequences in ribonuclease. *J. Biol. Chem.,* **207**: 201–210.

De Lamirande, G., and C. Allard, 1959. Studies on the distribution of intracellular ribonucleases. *Ann. N. Y. Acad. Sci.*, **81**: 570–584.

Dixon, M., and E. C. Webb, 1958. Enzymes. Academic Press, Inc., New York. 782 pp.

Edmonds, M., and J. S. Roth, 1960. The purification and properties of a ribonuclease from squid. *Arch. Biochem. Biophys.*, **89**: 207–212.

Fuglister, F. C., 1947. Average monthly sea surface temperatures of the Western North Atlantic Ocean. *Papers Phys. Oceanog. Meteorol. Mass. Inst. Tech. Woods Hole Oceanog. Inst.*, **10**: 2, 1–25.

Gowanloch, J. N., 1927. Contributions to the study of marine gastropods. II. The intertidal life of *Buccinum undatum*, a study in non-adaptation. *Contr. Canad. Biol.*, **3**: 167–178.

Johnson, C. W., 1934. List of marine Mollusca of the Atlantic coast from Labrador to Texas. *Proc. Boston Soc. Nat. Hist.*, **40**: 1, 1–204.

Kalnitsky, G., J. P. Hummel, H. Resnick, J. R. Carter, L. B. Barnett and C. Dierks, 1959. The relation of structure to enzymatic activity in ribonuclease. *Ann. N. Y. Acad. Sci.*, **81**: 542–569.

Kaplan, N. O., 1963. Symposium on multiple forms of enzymes and control mechanisms. 1. Multiple forms of enzymes. *Bact. Rev.*, **27**: 155–169.

Kenney, J. W., and A. G. Richards, 1955. Differences between leg and flight muscle of the giant water bug, *Lethocerus americanus*. *Ent. News*, **66**: 29–36.

Lin, S., and A. G. Richards, 1956. A comparison of two digestive enzymes in the house fly and American cockroach. *Ann. Ent. Soc. Amer.*, **49**: 239–241.

Maver, M. E., E. A. Peterson, H. A. Sober and A. E. Greco, 1959. Purification and characterization of ribonuclease of calf spleen. *Ann. N. Y. Acad. Sci.*, **81**: 599–610.

Mutchmor, J. A., and A. G. Richards, 1961. Low temperature tolerance of insects in relation to the influence of temperature on muscle apyrase activity. *J. Ins. Physiol.*, **7**: 141–158.

Read, K. R. H., 1964. Comparative biochemistry of adaptations of poikilotherms to the thermal environment. Proceedings of Symposium on Experimental Marine Ecology. *Occ. Publ.* No. 2, *Grad. School of Oceanography, Univ. R. I.*, 39–47.

Roth, J. S., 1959. Comparative studies on tissue ribonucleases. *Ann. N. Y. Acad. Sci.*, **81**: 611–617.

Roth, J. S., and D. Bachmurski, 1957. Studies on the distribution and properties of the ribonuclease system in marine forms. *Biol. Bull.*, **113**: 332.

Vroman, H. E., and J. R. C. Brown, 1963. Effect of temperature on the activity of succinic dehydrogenase from the livers of rats and frogs. *J. Cell. Comp. Physiol.*, **61**: 129–131.

Warmke, G. L., and R. T. Abbott, 1961. Caribbean Seashells. Livingston, Narberth. 346 pp.

HORMONAL CONTROL OF RESPIRATORY METABOLISM DURING GROWTH, REPRODUCTION, AND DIAPAUSE IN MALE ADULTS OF PYRRHOCORIS APTERUS L. (HEMIPTERA)[1]

K. SLÁMA[2]

Department of Insect Physiology, Entomological Institute,
Czechoslovak Academy of Sciences, Praha

In many species of insects the larval stages do not accumulate sufficient reserve materials to provide for egg maturation in the adult female. Such females may consume a considerable amount of food after emergence as adults and their body size and metabolic activity increase. In most of these cases nutrition and reproduction are under hormonal control. The corpus allatum hormone (CAH) activates the ovaries and the accessory glands of the reproductive tract; in the absence of the hormone, yolk deposition cannot proceed and the ovaries and accessory glands remain inactive (reviews by Wigglesworth, 1954; Pflugfelder, 1958; Novák, 1960). Furthermore, the activation hormone (AH) from the cerebral neurosecretory cells-corpora cardiaca may also be necessary for the deposition of yolk, apparently through its control over enzyme secretion in the alimentary tract (Thomsen and Møller, 1963).

In contrast to the females, however, growth and reproduction of the adult male appears to depend little, if at all, on hormones, even in those species in which hormones control the reproductive activities of the female. Thus, in most cases the process of spermatogenesis appears to be independent of hormones although the corpus allatum hormone may in some cases stimulate the growth and function of the accessory reproductive glands (Wigglesworth, 1936; Thomsen, 1942; Scharrer, 1946; Scharrer and von Harnack, 1958; Pflugfelder, 1958; Johansson, 1958). Janda and Sláma (1964) have shown that much less reserve material is metabolized during spermatogenesis than during egg maturation. The post-emergence somatic growth is therefore usually less in males than in females, as is the total metabolic activity. Similarly, the difference between active and diapause development is less apparent in males than it is in females (Ushatinskaya, 1957; Hodek and Čerkasov, 1963).

In an earlier paper Sláma (1964) showed that the respiratory rate in females of *Pyrrhocoris* is cyclical, increasing and decreasing periodically in close connection with the cycles of reproduction and oviposition. The cycles disappear after the corpus allatum or the ovaries are removed; the females then maintain a constant

---

[1] Acknowledgment is gratefully given to Dr. J. A. L. Watson, Department of Biology, Western Reserve University, Cleveland 6, Ohio, to Dr. B. Scharrer, Department of Anatomy, Albert Einstein College of Medicine, New York 61, New York, and to Prof. C. M. Williams, Department of Biology, Harvard University, who provided helpful comments on the typescript.

[2] Present address: Department of Biology, Harvard University, Cambridge, Mass.

intermediate rate of respiration. When the centers engaged in the release of the activation hormone (*i.e.,* the neurosecretory cells of the brain or the corpora cardiaca) are also removed, the postemergence growth of the females is suppressed and the respiration rate decreases to the level characteristic of diapause.

Sláma (1964) has therefore recognized three components in the respiratory metabolism of adult *Pyrrhocoris* females, each component having a characteristic response to hormones. The three components are: (a) The reproductive metabolism, which depends on the CAH or on the presence of ovaries; (b) digestive metabolism, which is independent of the CAH but depends on AH; and (c) basic metabolism of cells and muscle metabolism, which are not dependent on hormones and are identical with diapause metabolism. However, both the hormones may affect basal metabolism indirectly by activating specific tissues. Thus, the overall metabolism of an active female is controlled by the simultaneous cooperation of hormones; the CAH regulates metabolism in tissues associated with reproductive functions, whereas the AH regulates metabolism in tissues associated with trophic activity.

In the present study the relations between hormones and respiration have been investigated in adult males of *Pyrrhocoris.* The results are compared with findings obtained on female adults (see Sláma, 1964).

MATERIALS AND METHODS

Adult males of *Pyrrhocoris apterus* L. were used in all experiments. They were kept individually without females at 25° C. The techniques for rearing *Pyrrhocoris,* for operating on them, and for determining their oxygen consumption were described earlier (Sláma, 1964). The respiration rate of each specimen was measured at daily intervals at 25° C. The respiration data for specimens in which the operations were unsuccessful, or which died during the experiment were omitted from the final averages. At the end of each experiment the males were dissected and anatomical changes were noted.

RESULTS

1. *Growth and oxygen consumption of the normal male* (Fig. 1)

The process of spermatogenesis commences before the end of the last larval stage, and as early as two or three days after adult emergence the accessory glands have increased in size, the testes and the seminal vesicles contain spermatozoa and the male will mate. The increase in body weight is relatively small, and is restricted to the first three days of adult life; thereafter it remains constant at a level of about 55 or 60 mg. In females the body weight increases up to 80 mg. during the first 3 to 6 days of adult life, although the initial weight is about 40 to 55 mg. in both sexes. The considerable loss of weight which follows each oviposition in females is quickly compensated by a new intake of food.

The oxygen consumption was measured in a total of 20 males, three of which had pathologically swollen abdomens and were discarded. The remainder were used for Figure 1 b. For comparison, growth and oxygen consumption curves for normal females are given in Figure 1 a (from Sláma, 1964). A further 32 males were used to confirm the values for oxygen consumption at 15 days.

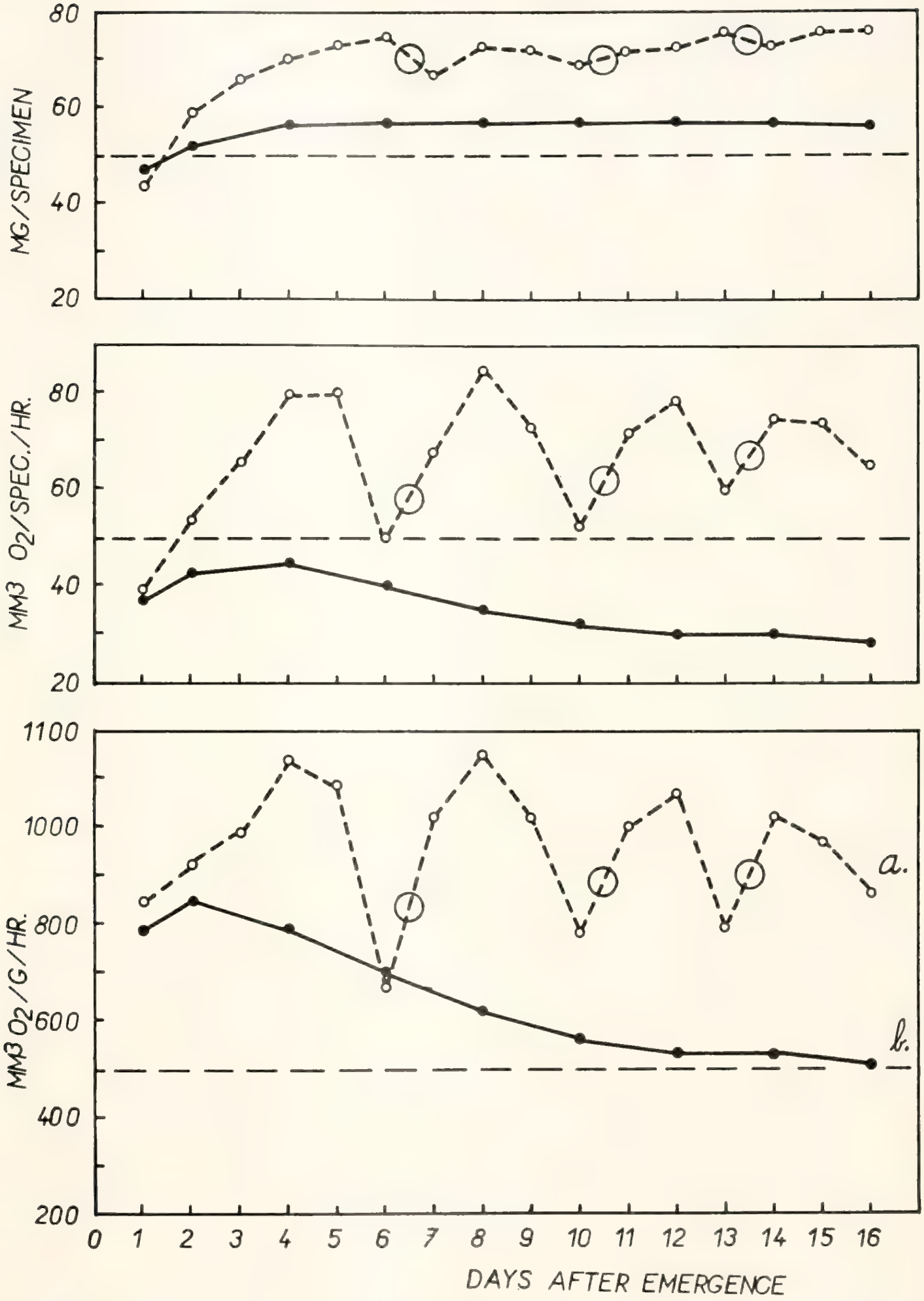


FIGURE 1. Changes in body weight (above), oxygen consumption per specimen (middle), and oxygen consumption per gram of the live weight (below) during adult life of normal females (a) (after Sláma, 1964), and of normal males of *Pyrrhocoris apterus* L. (b). Open rings indicate the moment of oviposition.

The oxygen consumption of the normal male appears to increase slowly during the growth period of the first three days; after this it diminishes steadily to stable values of about 400 to 500 mm.^{3}/gm./hr., where it remains (Fig. 1 b). There are no cycles of oxygen consumption in the normal males which would correspond to the cycles found in females. Furthermore, the rate of respiration in the normal male is very low when compared with that of the normal female; the maximal respiration rate in the female may be two or three times the stable rate of the male.

2. *Operated controls* (Fig. 2)

As a control experiment for the allatectomies and cardiac-allatectomies described below, ten males were subjected to narcosis and the operation through the neck membrane was performed, except that the glands were not removed (see Sláma, 1964). One of the operated animals died, and the remaining nine were used for Figure 2 c.

As Figure 2 c shows, the body weight and oxygen consumption of the operated males resemble those of the normal males. Thus, neither narcosis nor the neck membrane incision, *per se*, had an effect on growth and respiration.

3. *Allatectomized males* (Fig. 2 d)

Of 22 operated males, three died, and three had the corpus allatum incompletely removed; the remaining 16 were used for Figure 2 d. A further 25 allatectomized males were used to check the values of oxygen consumption at 15 days.

Anatomically, the allatectomized males differ little from the normal males. Their testes and seminal vesicles contain spermatozoa and they are able to mate. However, the accessory reproductive glands are subnormal in size in at least half of the allatectomized males.

The changes in the body weight of allatectomized males are similar to those of normal males or operated controls. The failure of the body weight to increase after allatectomy indicates that reserve material does not accumulate as it does in allatectomized females. During an initial period of about six days after emergence and operation, the oxygen consumption of allatectomized males appears slightly lower than that of normal males or controls. However, the later stable value for the respiration rate is similar to that in the other groups (Fig. 2 d). The oxygen consumption of the 25 additional allatectomized males was consistent with the figures given in Figure 2 d, the average being 470 mm.^{3} O_2/gm./hr.

From these results it is evident that the removal of the corpus allatum has little effect on the respiratory metabolism of *Pyrrhocoris* males. This contrasts with the situation in females, where allatectomy considerably depresses the rate of respiration.

4. *Cardiac-allatectomized males*

Of 15 cardiac-allatectomized males, three died, one had the corpora cardiaca incompletely removed and 11 survived. The weight and oxygen consumption were measured only at the first, fourth, twelfth and seventeenth days after emer-

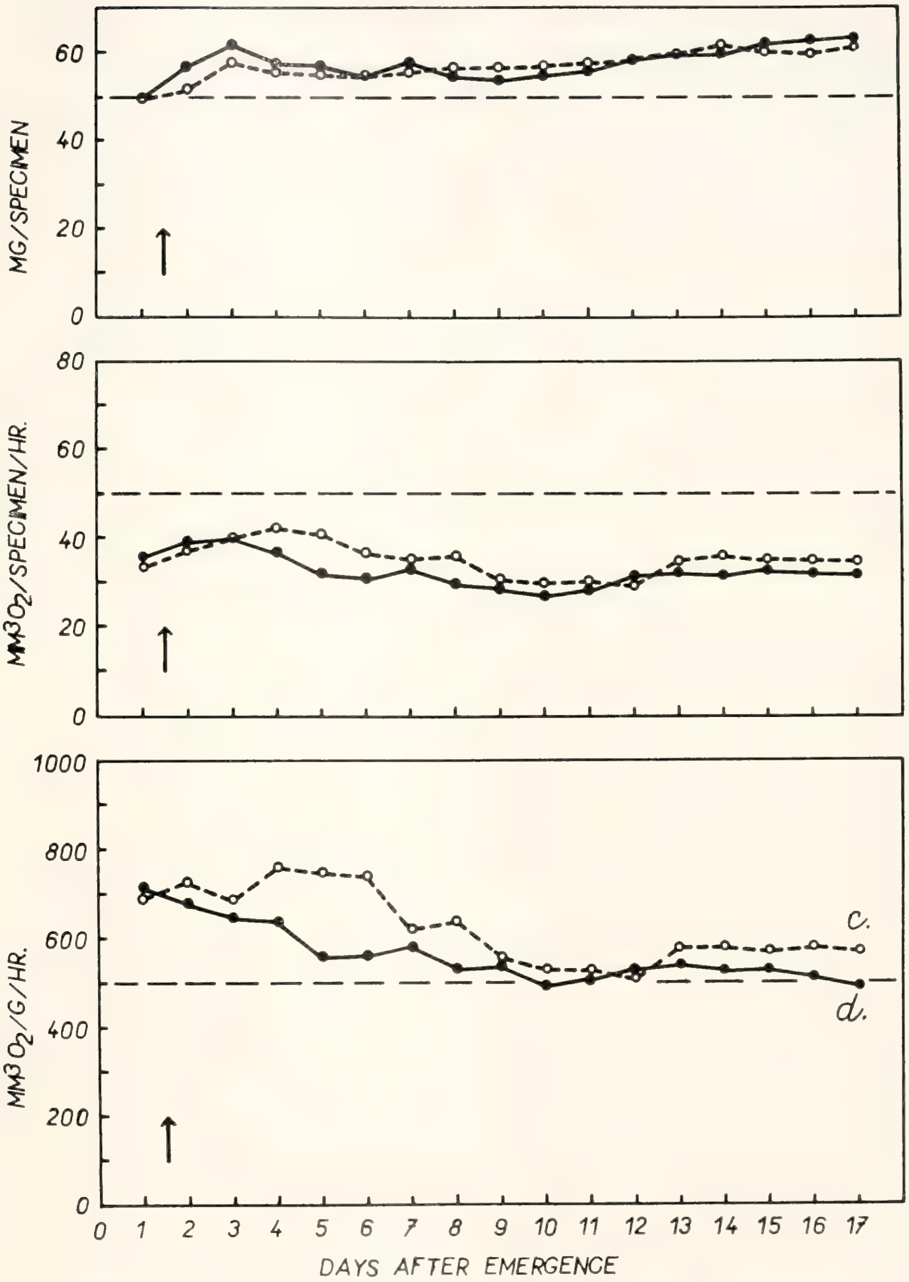


FIGURE 2. Changes in body weight (above), oxygen consumption per specimen (middle), and oxygen consumption per gram of live weight (below) during adult life of sham-operated (c) and allatectomized (d) males of *Pyrrhocoris*. Arrows indicate the moment of operation.

gence and operation. The rate of respiration at 15 days was measured on an additional series of 18 cardiac-allatectomized males.

The changes in the rates of growth and oxygen consumption during adult life in cardiac-allatectomized males were again similar to those of normal or allatectomized males. The additional values at 15 days, averaging 452 mm.^{3}/gm./hr., confirm that the stable rate of respiration in cardiac-allatectomized males does not differ from that in the other experimental groups.

The results show that not only allatectomy but also cardiac-allatectomy has little if any effect on the respiration rate of *Pyrrhocoris* males. This again contrasts sharply with the situation in females, where cardiac-allatectomy reduces the respiration rate to as little as one third of the normal level. However, the rate of respiration in cardiac-allatectomized or normal males is very similar to that of cardiac-allatectomized females.

5. *Castrated males* (Fig. 3)

Of 15 males that emerged from larvae castrated in the last instar, two died and two were incompletely castrated. The remaining 11 were used for Figure 3 e. Additional measurements at 15 days were taken on a series of 17 castrated males.

The curves for growth and respiration of the castrated males are again similar to those of the other males (Fig. 3 e), as are the values at 15 days. These results show that the respiratory metabolism of testicular tissue is insignificant relative to the metabolism of the rest of the animal. In the adult female, on the other hand, the removal of the ovaries results in a depression of the respiration rate.

6. *Diapausing males* (Fig. 3)

To induce diapause, males were kept under a short photoperiod (8 hours of light per day) from the beginning of the 5th instar; the experimental conditions were otherwise unaltered. Measurements of 11 diapausing males with undeveloped accessory glands were used for Figure 3 f. Specimens in which these glands had been found developed normally after dissections were omitted.

Morphologically, the diapausing and active males are closely similar but the accessory glands of the former are usually less developed and the potential for mating is limited. The growth and respiration curves of diapausing males again follow the course common to the other experimental series. In diapausing females, on the other hand, respiration is greatly suppressed, reaching levels that are very similar to the respiration rates of the males. Thus, as far as respiration is concerned one may consider all the males (whether normal, allatectomized, cardiac-allatectomized or castrated) to be in "diapause" when compared with females.

7. *The effect of mating on respiration in the male* (Fig. 4)

The data in the preceding sections have shown that the respiratory metabolism of the males, unlike that of the females, is largely independent of hormones or gonads. Because none of the males was allowed to mate, it could be argued that

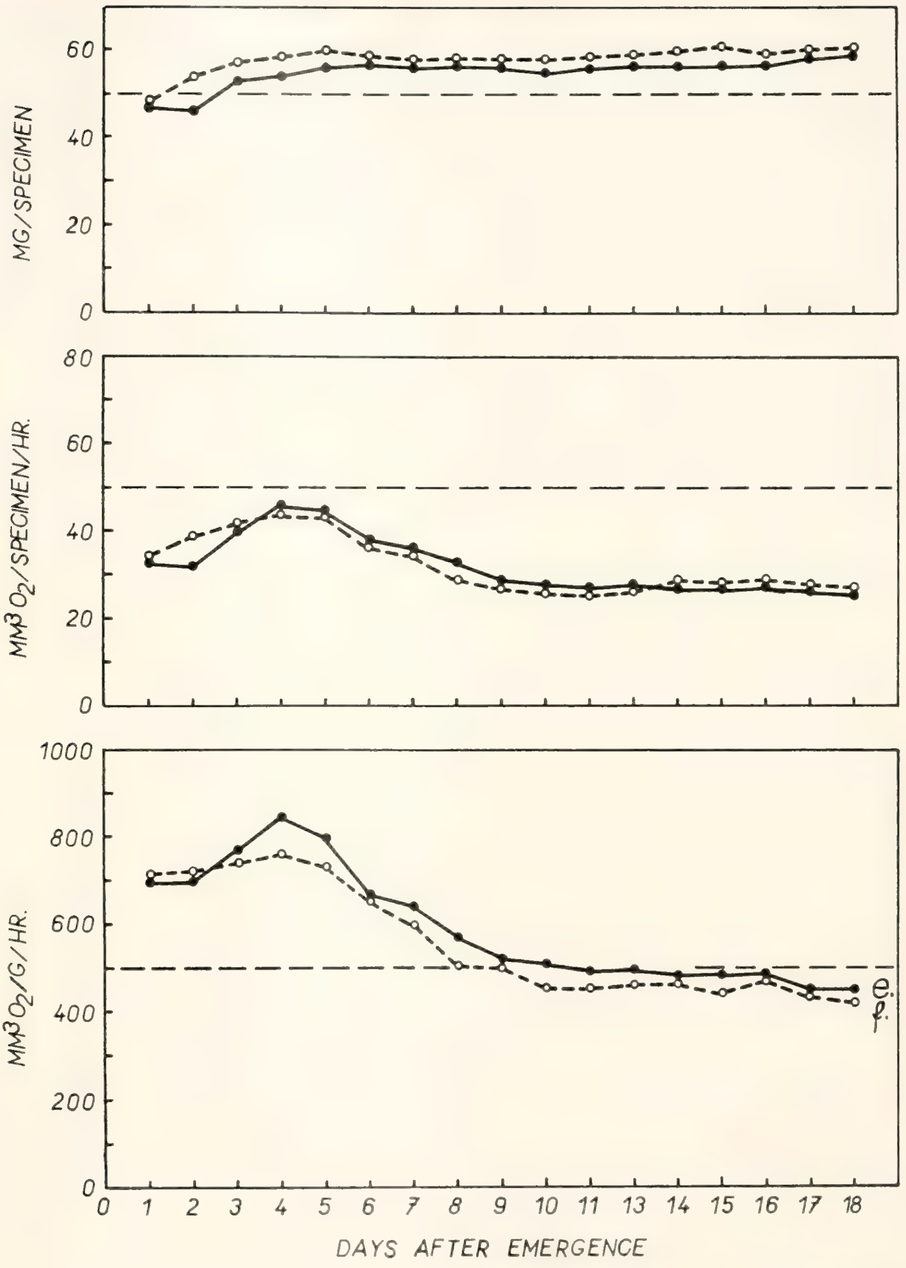


FIGURE 3. Changes in body weight (above), oxygen consumption per specimen (middle), and oxygen consumption per gram of live weight (below) during adult life of castrated (e), and diapausing (f) males of *Pyrrhocoris*.

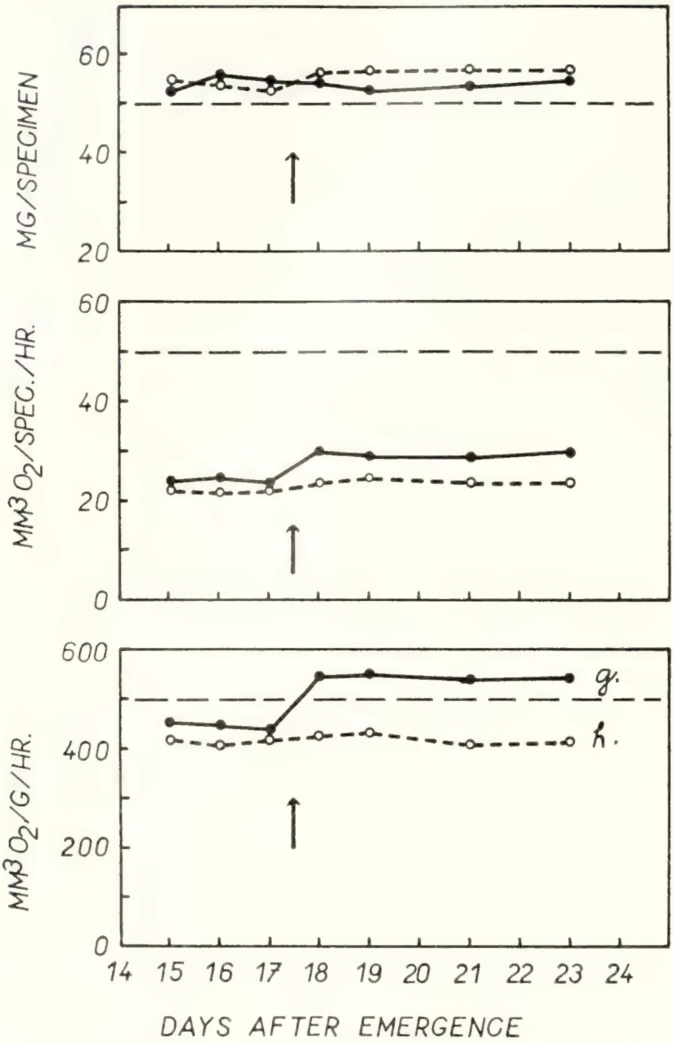


FIGURE 4. Changes in body weight (above), oxygen consumption per specimen (middle), and oxygen consumption per gram of live weight (below) of normal males (g), and allatectomized (h) males of *Pyrrhocoris* which were allowed to mate. Arrows indicate the beginning of mating [illegible]

the apparent incompetence is a consequence of sexual inactivity. To clarify this point, the rate of respiration was measured in 11 intact males, aged 15 days, first without females, and then after the males had mated with up to three females. After such intensive mating the rate of respiration increases about 20% (Fig. 4 g). It is possible that the increase is connected with the synthesis of material in the depleted accessory glands. Similar experiments therefore were carried out on 11 allatectomized males also aged 15 days. The mating activity in this case was not followed by increase in the rate of respiration (Fig. 4 h). Furthermore, the size of the accessory glands of allatectomized males increases very little after mating. These results support the assumption that the effect of intensive mating on the respiration of the normal males is connected with changes in the synthetic activity of the accessory glands.

8. *The effect of feeding on the respiration of males*

Adult males of *Pyrrhocoris* consume relatively little food when compared with females. As the digestion and utilization of food influence the respiration of adult females, it was necessary to determine the extent to which the respiration of males depends on these factors. Eleven males were therefore starved for three months in a refrigerator at 10° C., and their rates of respiration were measured. They were then transferred to 25° C., their respiration was remeasured, and they were then fed. The rate of respiration in these males increased after feeding; the oxygen consumption at 25° C. rose from approximately 330 mm.^{3}/gm./hr. to 500 mm.^{3}/gm./hr. after feeding. Thus, processes associated with feeding influence the respiratory metabolism of males, although the effect is minor when compared with that found in females (see Sláma, 1960). The results also show that prolonged starvation decreases the respiratory rate of males, as it does in females; presumably, the effect depends on the reduction of metabolic processes that are normally independent of hormones.

9. *The effect of implanting female corpora allata on the respiration of males*

The independence of respiration on hormones in male *Pyrrhocoris* might be due to one of two factors: (a) The corpus allatum may be inactive. Certainly the corpora allata of male *Pyrrhocoris* are much smaller than those of the female in which the glands secrete actively. (b) The presumptive target organ (*e.g.*, the reproductive system) may not respond to the CAH.

To test these possibilities, each of 10 male *Pyrrhocoris,* 15 days old, was implanted with a corpus cardiacum-corpus allatum complex from a single active female; such an implantation into diapausing or cardiac-allatectomized females causes a rapid increase in oxygen consumption and in body weight (Sláma and Hrubešová, 1963). The oxygen consumption was measured before the operation and on the third and fifth days thereafter. Eight specimens survived. The results showed that the operation was followed neither by increased oxygen consumption nor by an increase in body weight, confirming that the hormones released from the corpora allata or the corpora cardica act only on specific tissues; effects on general metabolism, as in the females, are indirect. Thus, even a high concentration of hormone may be inactive metabolically if there are no tissues which are able to respond.

DISCUSSION

In females of various insect species, allatectomy leads to a decrease in oxygen consumption, whereas implantation of the active corpus allatum causes an increase (see Sláma, 1964). On the other hand, the present results show that the respiratory metabolism of males is largely or completely independent of hormonal activity.

Similar results have been obtained in *Calliphora*, where the implantation of extra corpora allata has no effect on respiration in the male, although it increases oxygen consumption in the female. Allatectomy also has less effect on the respiration of male *Calliphora* than it does on that of the female (Thomsen, 1949). Furthermore, the cytochrome oxidase activity in male *Drosophila* is independent of hormones (Bodenstein and Sacktor, 1952).

The respiration rate of male *Pyrrhocoris* is only a third to a half that of the active female (see Sláma, 1964). Two of the three components of respiratory metabolism which occur in females have no counterpart in males, *i.e.*, the active reproductive metabolism and digestive metabolism. One may therefore conclude that the respiratory metabolism of males is equivalent to the third type of female metabolism, *i.e.*, the basic metabolism of cells and muscles, or the metabolism of diapause. This component has a common characteristic in both sexes; it is independent of hormones and it is most evident when growth, digestion and reproduction are depressed to a minimum. This type of respiration is characterized by an oxygen consumption of about 20 to 25 mm.^{3}/hr./specimen in normal males and in females from which the corpora cardiaca and corpora allata had been removed (Sláma, 1964).

In the females the CAH regulates reproductive metabolism, probably by direct control over the ovarian tissue. The AH, on the other hand, regulates digestive metabolism, which is also probably a direct effect on the alimentary tract. This may explain the similarity between the respiratory metabolism of the normal male and the low metabolism of the females deprived of hormonal sources. The metabolic activity associated with reproduction and digestion is reduced so greatly in the normal male that any action of the hormones on overall metabolism is correspondingly reduced. In females Janda and Sláma (1964) have shown that when the activation hormone and digestion are working in the absence of the CAH or ovarian growth (as in allatectomized females), digestive activity leads to an enormous accumulation of reserve products. This is, however, not the case in males, where the metabolism of the intestinal tissue is little stimulated by the AH. These facts indicate that the total capability and the sensitivities of appropriate tissues are critical factors to be considered in the overall physiological interpretation of hormone action.

The critical difference between male and female metabolism in *Pyrrhocoris* therefore depends on the difference in the demands of the reproductive systems; the metabolism involved in basal maintenance is probably similar in the two sexes. In the female the loss of materials with the eggs, which may involve up to a third of the body weight, must be compensated by additional ingestion and digestion. As Sláma and Hrubešová (1963) have shown, the total amount of oxygen consumed for the production of all maturing eggs is very large. In the male, on the other hand, production of the spermatozoids involves the use of little reserve material, and in consequence castration has little effect on respiration. There is not much change in the size of the testes during maturation, and the major metabolic outlay arises most probably from the secretory activity of the accessory glands.

It is with the latter activity that the "reproductive metabolism" of the male is concerned, and this is a common phenomenon in insects. The CAH stimulates secretory activity in the accessory glands of *Rhodnius* (Wigglesworth, 1936), in

Calliphora (Thomsen, 1942), in *Schistocerca* (Loher, 1960), etc. Clearly, though, the glands in *Pyrrhocoris* are too small for hormonally induced changes in their activity to influence respiratory activity as a whole; it is only after gross depletion that such changes can affect respiration.

At present it is not possible to extrapolate from the results obtained on *Pyrrhocoris* to males of other insects. Stable and low levels of respiration have been found in adult males of *Leucophaea* in which the females have cyclical respiratory activity which may surpass that of the male (Sägesser, 1960). Yet in some species the respiration rate of males is higher than that of females, as in *Locusta* (Butler and Innes, 1936). In *Calliphora* (Thomsen, 1949) and in *Leptinotarsa* (Ushatinskaya, 1957) the respiration of both sexes is approximately equal. These differences cannot be evaluated without considering the processes which may contribute to them, such as the specific differences in the activity of the accessory glands, reproductive system, locomotor activity and the amount of inert reserve material.

The adult life of *Pyrrhocoris* males may last from two months to one year, depending mostly on their sexual activity. Except the brief time interval after adult ecdysis, the males keep a constant body size, reduced food ingestion, and low and steady respiration rate with uniform locomotory activity. This makes the male resistant against unfavored conditions of life, starvation, etc. Evidently, the main biological function of these males depends merely in their preservation for the purpose of female insemination. The induction of diapause has, therefore, little, if any effect on growth, respiration rate, locomotory activity or food ingestion rate, the differences between the normal and the diapausing males being restricted to the changes that occur in the accessory sexual glands. These observations may also demonstrate the lack of the hormonal stimulation of metabolism in the males of *Pyrrhocoris,* because diapause in insects is generally considered as a result of hormonal deficiency. In females of *Pyrrhocoris,* where the hormonal regulation of metabolic processes appears to be apparent, the induction of diapause is always followed by a suppression of growth and metabolism (Sláma, 1964).

SUMMARY

The respiratory metabolism of adult *Pyrrhocoris* males is very low when compared with that of females, and it is independent of hormones. Allatectomy, cardiac-allatectomy, castration and diapause have no profound effect on the rate of respiration in the adult male, unlike the situation in the adult female. Indeed, males respire at about the same rate as females deprived of endocrine glands. The sexual dimorphism in respiration and hormone action is correlated with the differences in growth, digestion and reproduction. It is shown that the hormones influence the over-all metabolism indirectly by regulating the morphology and physiological activity of specific tissues.

LITERATURE CITED

Bodenstein, D., and B. Sacktor, 1952. Cytochrome c oxidase activity during the metamorphosis of *Drosophila virilis*. *Science*, **116**: 299–300.

Butler, C. G., and J. M. Innes, 1936. A comparison of the rate of metabolic activity in the solitary and migratory phases of *Locusta migratoria*. *Proc. Roy. Soc. London, Ser. B*, **119**: 296–304.

HODEK, I., AND J. ČERKASOV, 1963. Imaginal dormancy in *Semiadalia undecimnotata* Schneid. II. Changes in water, fat and glycogen content. *Acta Soc. Zool. Bohemoslov.*, **27**: 298–318.

JANDA, V., JR., AND K. SLÁMA, 1964. Einfluss von Hormonen auf den Glykogen-, Fett-, und Stickstoffmetabolismus bei Adulten *Pyrrhocoris apterus* L. (Hemiptera). *Zool. Jahrb. Physiol.*, **70** (in press).

JOHANSSON, A. S., 1958. Relation of nutrition to endocrine-reproductive functions in the milkweed bug *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae). *Nytt Mag. Zool.*, **7**: 1–132.

LOHER, W., 1960. The chemical acceleration of the maturation process and its hormonal control in the male of the desert locust. *Proc. Roy. Soc. London, Ser. B*, **153**: 380–397.

NOVÁK, V. J. A., 1960. Insektenhormone (2nd Ed.), NČSAV, Prague.

PFLUGFELDER, O., 1958. Entwicklungsphysiologie der Insekten (2nd Ed.). Akademische Verlagsgesellschaft, Leipzig.

SÄGESSER, H., 1960. Über die Wirkung der Corpora allata auf den Sauerstoffverbrauch bei der Schabe *Leucophaea maderae* (F). *J. Ins. Physiol.*, **5**: 264–285.

SCHARRER, B., 1946. The relationship between corpora allata and reproductive organs in adult *Leucophaea maderae* (Orthoptera). *Endocrinology*, **38**: 46–55.

SCHARRER, B., AND M. VON HARNACK, 1958. Histophysiological studies on the corpus allatum of *Leucophaea maderae*. I. Normal life cycle in male and female adults. *Biol. Bull.*, **115**: 508–520.

SLÁMA, K., 1960. Oxygen consumption during the postembryonic development of *Pyrrhocoris apterus* and its comparison with that of Holometabola. *Ann. Ent. Soc. Amer.*, **53**: 606–610.

SLÁMA, K., 1964. Hormonal control of respiratory metabolism during growth, reproduction, and diapause in female adults of *Pyrrhocoris apterus* L. (Hemiptera). *J. Ins. Physiol.*, **10**: 283–303.

SLÁMA, K., AND H. HRUBEŠOVÁ, 1963. Übereinstimmung in der Einwirkung von larvalen und imaginalen Corpora allata auf den Respirationsmetabolismus und die Reproduktion bei *Pyrrhocoris apterus* L. Weibchen. *Zool. Jahrb. Physiol.*, **70**: 291–300.

THOMSEN, E., 1942. An experimental and anatomical study of the corpus allatum in the blowfly, *Calliphora erythrocephala* Meig. *Vidensk. Medd. naturh. Foren. Kbh.*, **106**: 320–405.

THOMSEN, E., 1949. Influence of the corpus allatum on the oxygen consumption of adult *Calliphora erythrocephala* Meig. *J. Exp. Biol.*, **26**: 137–149.

THOMSEN, E., AND I. MØLLER, 1963. Influence of neurosecretory cells and of corpus allatum on intestinal protease activity in the adult *Calliphora erythrocephala* Meig. *J. Exp. Biol.*, **40**: 301–321.

USHATINSKAYA, R. S., 1957. Osnovy kholodostoikosti nasekomykh. Izdatelstvo Akademii Nauk SSSR, Moscow.

WIGGLESWORTH, V. B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.*, **79**: 91–121.

WIGGLESWORTH, V. B., 1954. The Physiology of Insect Metamorphosis. Cambridge University Press, Cambridge.

PHYSIOLOGY OF INSECT DIAPAUSE. XIV. AN ENDOCRINE MECHANISM FOR THE PHOTOPERIODIC CONTROL OF PUPAL DIAPAUSE IN THE OAK SILKWORM, ANTHERAEA PERNYI

CARROLL M. WILLIAMS[1] AND PERRY L. ADKISSON[2]

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

During the final ten days of larval life, the Pernyi silkworm envelops itself in a stout-walled cocoon within which it pupates. Development may stop right there as the pupa begins a prolonged period of pupal diapause which persists until the following spring. Alternatively, the newly formed pupa may develop into an adult moth without any delay. The moth is then committed to the reproduction of a further generation of pupae which can begin to diapause before the first killing frost. If winter arrives before the larvae can pupate, the insect will experience what is little short of "ecological suicide."

Like so many plants and animals, the Pernyi silkworm minimizes these ecological dangers by monitoring seasonal signals of utmost precision, namely, the lengths of the night and day. For *A. pernyi* the phenomenon is well documented in the detailed studies of the Japanese investigator, Tanaka (1950a, 1950b, 1950c; 1951a, 1951b; see Lees, 1955, for English summary). Thus, when Pernyi larvae are reared under day-lengths longer than 14 hours, they develop without any pupal diapause; at temperate latitudes, photoperiods of this sort are peculiar to late spring and early summer when the season is propitious for a second brood. By contrast, larvae reared under day-lengths shorter than 14 hours (as in late summer and autumn) transform into diapausing pupae.

Little is known about the physiological basis of the photoperiod response. In principle, the minimal mechanism must include, not only a photoreceptor, but also a clockwork-computer which counts the hours of darkness and daylight. Until recently, all that was known was Tanaka's finding that the larval ocelli are not involved in the reception of photoperiod.

But the induction of diapause is only half the story. Of equal significance is the termination of diapause—its timing and synchronization with the seasons.

In a related species, the Cecropia silkworm, the termination of pupal diapause is known to be controlled primarily by environmental temperature. By an apparently direct action on the brain itself, environmental temperature conditions the secretion of a hormone prerequisite for the termination of diapause and the initiation of adult development (Williams, 1956).

This same temperature-sensitive system has generally been presumed for other diapausing pupae including *A. pernyi.* Indeed, as pointed out in de Wilde's

---

[1] This study was supported, in part, by a grant from the National Science Foundation.

[2] Special Postdoctoral Fellow of the U. S. Public Health Service during the term of the study. Permanent address: Department of Entomology, Texas A & M University, College Station, Texas.

(1962) recent review, insect pupae (unlike all other stages in the life history) are thought to be insensitive to photoperiod.

Some reservations on this point are provoked by a more detailed examination of *A. pernyi.* Thus, when the cocoon is cut open, the pupa is always facing upward in the chamber. Moreover, on inspecting the pupa, itself, one cannot fail to be impressed by the unpigmented, transparent cuticle overlying the brain (Fig. 1). Even if the rest of the pupa is jet-black, the facial cuticle is always pigment-free. By moistening it with alcohol, one can look inside and see the underlying brain. A similar "facial window" is found in all pupae of the genus *Antheraea,* including the American species, *A. polyphemus.*

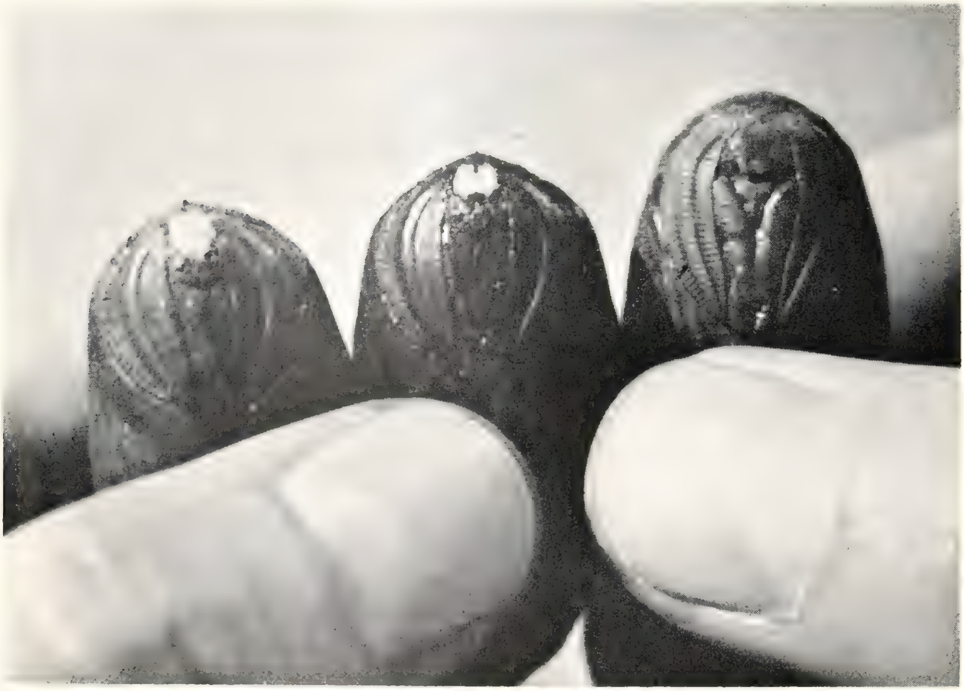


FIGURE 1. Two pupae of *A. pernyi* are here photographed alongside a Cecropia pupa. Pernyi routinely shows a zone of transparent cuticle overlying the pupal brain; the Cecropia pupa does not.

Is it possible that the window has something to do with the transmission of light? This prospect seems to have been reported only by the Russian investigator, Shakhbazov (1961).

Impressed by the facial window, one of us tested, some 16 years ago, the influence of illumination on diapausing pupae of *A. polyphemus.* Groups of pupae were removed from cocoons, subdivided into two lots, and placed at 25° C. in continuous light and darkness, respectively. Illumination had no detectable effects on the rate of termination of diapause.

Subsequently we have learned that continuous light or darkness are both aperiodic signals which, in this sense, are inappropriate tests for photoperiodism. The experiment has now been repeated in a proper manner by making use of specific photoperiods. Certain of the results have been summarized previously (Williams, 1963; Williams and Adkisson, 1964).

MATERIALS AND METHODS

1. *Experimental animals*

All experiments were performed on pupae of *A. pernyi.* The cocoons were the diapausing first-brood harvested in late July; on August 27 they were shipped from Japan in a series of opaque cardboard boxes. They arrived at Harvard University on September 30.

One hundred pupae were removed from cocoons and inspected under the dissecting microscope to confirm the persistence of diapause. The rest of the cocoons were then subdivided into two lots. One group was spread on tables in a 25 ± 0.5° C. room programmed for a daily illumination of 8 hours; the other group was stored in opaque boxes at 2–3° C.

A few experiments were performed on a subsequent shipment consisting of diapausing cocoons of the second generation harvested in October. This material was stored at 2–3° C.

2. *Photoperiod studies*

Chilled and unchilled cocoons were exposed for 16 weeks to precise regimens of photoperiod. Some pupae were removed from their cocoons at the outset of the experiment; the vast majority were not.

After the insects had been placed at the selected photoperiods, they were inspected at least twice weekly, the number of emerged moths being recorded. Pupae not enclosed in cocoons were examined under the dissecting microscope to ascertain whether adult development had begun. Needless to say, all observations (with the exception of the individuals maintained under continuous darkness) were performed during the photophase of the daily cycle. Chambers maintained in continuous darkness were opened in a dark room and inspected under red light.

3. *Adult development*

The initiation of adult development is signaled by the detachment and retraction of the epidermis from the pupal cuticle. The "zero day of development" was recognized by the initiation of retraction of the epidermis on the ventral side of the tip of the abdomen where the cuticle overlying the genital disc is usually palely pigmented. The overlying cuticle was moistened with 70% ethanol and viewed under a dissecting microscope. In order to eliminate surface reflections, observations of this type are facilitated if a Polaroid filter is placed in front of the microscope lamp and a second "crossed" filter is positioned under the objective lens (Harvey and Williams, 1958).

In pupae of pale pigmentation, the initiation of epidermal retraction was also visible in the pupal wings at the onset of adult development. But in the jet-black pupae which are sometimes encountered, observation of wing retraction was possible only when a zone of the superficial pigment layer was scraped away with a scalpel. A time-table for the adult development of Pernyi will be described in the section on Results.

4. *Experimental chambers*

Two types of chambers were employed. The first consisted of six B.O.D. incubators modified so that each of the three shelves was illuminated from directly overhead by a 15-watt fluorescent lamp (Sylvania cool white, F15T8). The average intensity of illumination was approximately 175 foot-candles (1883 lux).

The second type of chamber was an adaptation of that described by Dutky *et al.* (1962). Five-gallon tin-cans with tight-fitting lids were employed. A 4-watt fluorescent lamp (General Electric cool white F4T5) was installed in the lid of each can to yield an average internal illumination of 110 foot-candles (1184 lux). An electrically driven exhaust fan was attached to the lid and an air-intake placed near the bottom; the intake and exit ports were fitted with coiled lengths of radiator hose to prevent any leakage of light. The assembled chambers were placed in a constant-temperature room at 25 ± 0.5° C. and a relative humidity of 60%. It may be noted that these simple chambers were in every way as satisfactory as the expensive B.O.D. incubators.

The daily cycle of illumination was programmed for each chamber by a Model No. 8001 "Tork" time-clock. In the case of the B.O.D. incubators, the timer's double-throw switch was used to turn on a 50-watt heater (positioned in the rear of the lower shelf) whenever the fluorescent lamps were turned off. This arrangement minimized the temperature fluctuations occasioned by the operation of the lamps.

In all cases (except, of course, the chambers maintained in continuous light or dark) the chambers were programmed for 24-hour daily cycles of light and darkness. For convenience, we shall identify each photoperiod in terms of the duration of the daily light-cycle or "photophase." By so doing, we automatically define the duration of dark-cycle or "scotophase." This terminology seems most straight-forward despite the fact that the length of the night is probably more crucial than the length of the day (Adkisson, 1964).

5. *Photoperiod gradients*

In order to expose opposite ends of individual pupae to different photoperiods, two simple mechanisms were utilized. The first of these consisted of a block of wood in which a series of circular holes, 15 mm. and 17 mm. in diameter, was drilled. A second board was screwed to the bottom of the first to seal the lower ends of the holes. All surfaces were painted with a non-reflecting black paint.

Pupae were selected of appropriate diameters to slip snugly into the holes by friction-fit. Half faced upwards in the holes; half downwards. The entire assembly was placed in a 25° C. incubator programmed for an 8-hour photophase. In this manner one end of each individual was exposed to the 8-hour photophase while the opposite end was maintained in continuous darkness.

In later experiments the set-up was modified to permit one to establish specific photophases on both upper and lower surfaces. For this purpose six rows of holes were drilled through a 15 × 40 × 4 cm. board. The top side was framed to receive a tight-fitting removable lid and all surfaces were painted black.

As described above, the holes were plugged with pupae and the entire assembly placed in a 25° C. incubator programmed for a 16-hour daily photophase. With the lid removed, both upper and lower surfaces were illuminated by the incubator's fluorescent lamps above and below the assembly. After 8 hours of the 16-hour photophase, the lid was sealed in place. Sixteen hours later, at the beginning of the next photophase, the lid was removed. This cycle was repeated daily. The net effect was to expose the upward-facing ends to an 8-hour photophase while the downward-facing ends of the same individuals received a 16-hour photophase.

EXPERIMENTAL RESULTS

1. *Time-table for adult development at 25° C.*

As described under Methods, the "zero day of adult development" was recognized in terms of the initiation of retraction of the epidermis. We would emphasize that regardless of how many days, weeks, or months are required for the initiation of adult development, the latter, once begun, then proceeds at a rate dictated almost entirely by environmental temperatures. (See below under 7C.)

In Table I we present an abridged version of a time-table for adult development at 25° C. Characters singled out are in most cases visible in the intact animal under the dissecting microscope. When maintained at 25° C., the moths emerged 19 ± 2 days after the visible initiation of development. The latter, as signaled

TABLE I

Time-table for the adult development of Antheraea pernyi at 25°C, with special reference to externally visible characters

Day	Characters
0	The epidermis begins to retract from the overlying pupal cuticle in the wings and at the underside of the tip of abdomen; no visible retraction of leg epidermis.
1	Full retraction of the epidermis of wings and tip of abdomen; facial retraction present only along posterior margin and in posterior angles; trace of leg retraction.
2	Full retraction of leg epidermis.
7	Compound eyes fully faceted and show initiation of pale pink pigmentation; genitalia fully formed but show no silky pubescence.
9	Brown pigmentation of eyes. Genitalia covered with silky pubescence but cuticle remains unpigmented.
10	Dark brown pigmentation of eyes; genitalia remain covered with silky hairs and cuticle remains unpigmented; no pigmentation of tarsal claws.
11	Black pigmentation of tarsal claws.
12	Coarse white hairs are seen for the first time.
14	No pigmentation of wings; coarse white hairs still present on face and genitalia.
15	Pigment appears in "eye spots" of forewings; animal not "soft."
16	Full pigmentation of wings; animal begins to soften due to breakdown of pupal endocuticle.
18	Animal soft; wings fully pigmented; molting fluid still present.
19	Resorption of molting fluid begins.
20	Molting fluid resorbed and replaced by air; elongation and distension of body; moth emerges and expands wings.

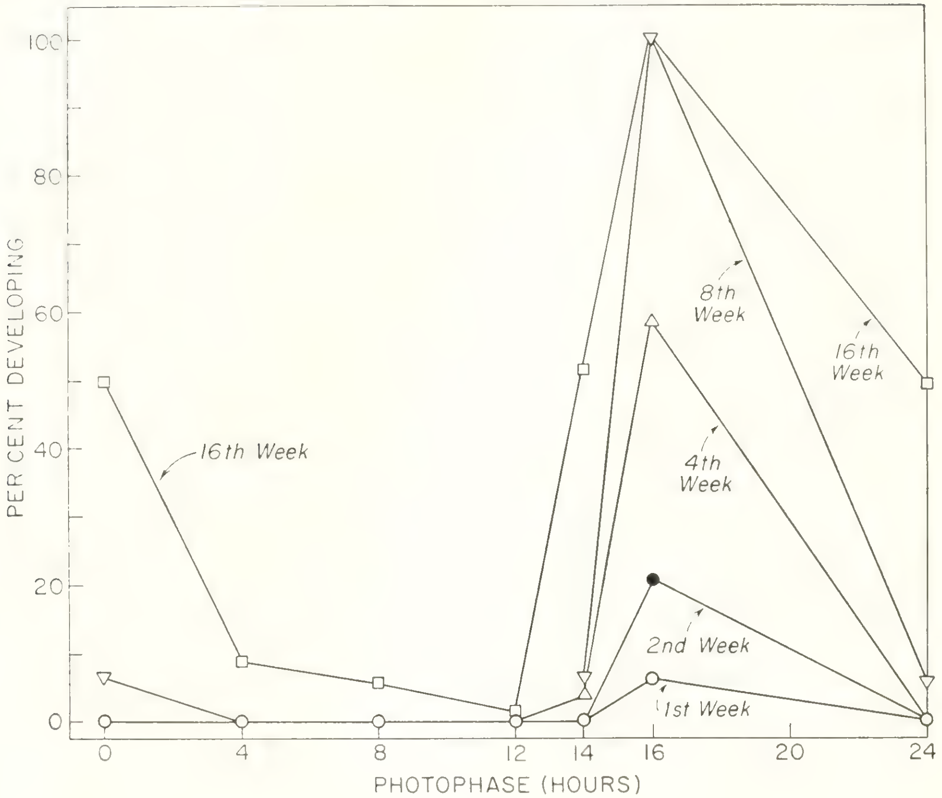


FIGURE 2. The effects of photoperiod on the termination of diapause by unchilled pupae of *A. pernyi* at 25° C. The termination of diapause was recognized or computed in terms of the zero day of adult development.

by the initiation of epidermal retraction, may be recognized with a precision of 4 to 6 hours.

A similar calibration of the diapausing second-brood pupae revealed a slightly faster pace of adult development in that the moths emerged 17 ± 2 days after the visible initiation of development.

2. *The influence of photoperiod on the termination of diapause*

A. *Unchilled pupae*

Groups of 100 cocoons and 48 naked pupae were placed at seven different photoperiods at 25° C. The results, summarized in Figure 2, reveal that the 16-hour photophase was most effective in promoting the termination of diapause; after only 4 weeks, over 50% of these individuals showed the initiation of adult development. By contrast, diapause was persistent in the presence of short-day regimens. A 12-hour photophase was especially effective and sustained the diapause of 98% of pupae during the test period of 16 weeks. Attention is also directed to the similar effects of prolonged exposure to continuous light or darkness. Finally, we can state the surprising finding that the response to photoperiod was the same for naked pupae and for those which remained in cocoons.

B. *Chilled pupae*

The preceding experiment was repeated in greater detail, using previously chilled pupae. These individuals were stored at 2–3° C. from the first week of October; for this series of experiments they were removed from storage between November 30 and January 7 and used immediately. Groups of 50 cocoons were placed at ten different photoperiods at 25° C.

The results, recorded in Figure 3, were essentially the same as observed for unchilled pupae, the only major difference being an accelerated response to the regimens which terminated diapause. With these additional data, we now see that the most effective stimulus for the termination of diapause is provided by a 17-hour photophase. And, here again, the 12-hour photophase was most effective in preventing the termination of diapause.

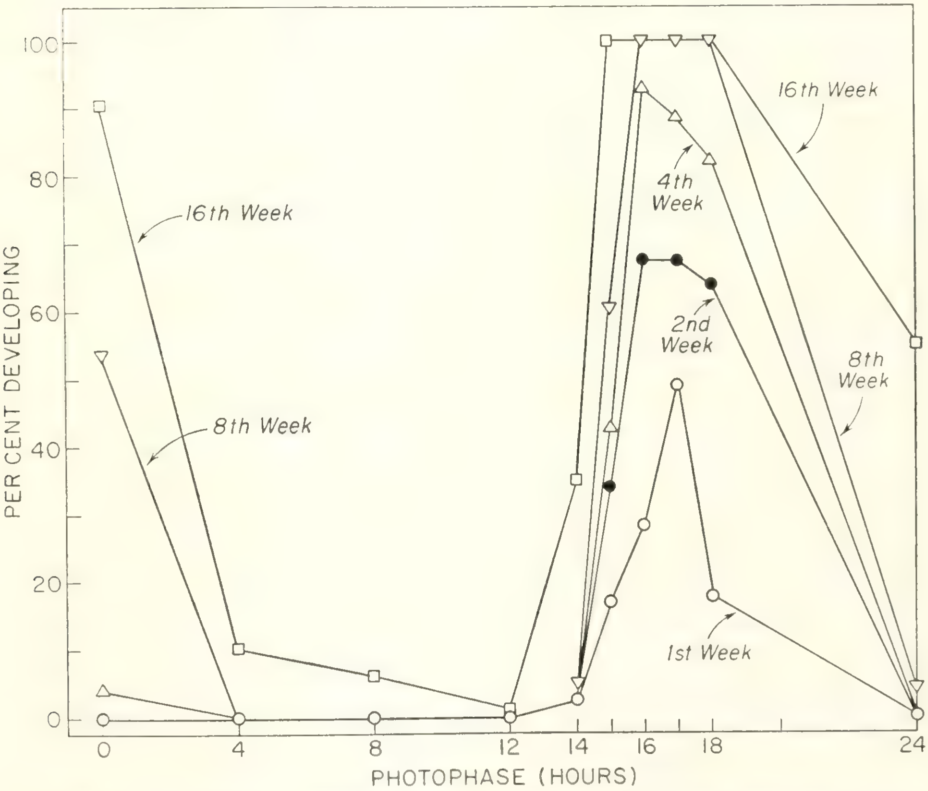


FIGURE 3. The effects of photoperiod on the termination of diapause by previously chilled pupae of *A. pernyi* at 25° C. Diapause is most persistent in the presence of a daily photophase of 12 hours; it is most promptly terminated by a photophase of 17 hours.

3. *The "fine structure" of the photoperiod response*

As is amply demonstrated in Figures 2 and 3, an abrupt transition between "short-" and "long-day" conditions occurs at or near a photophase of 14 hours. This finding was examined in further detail by exposing two groups of 50 unchilled pupae to daily photophases of 13.50 and 13.75 hours, respectively. At the end of eight weeks, 2% of the former group and 22% of the latter group had initiated adult development. This difference signals a discrimination between photophases differing by only 15 minutes.

4. *Effects of preliminary exposure to an 8-hour photophase*

In section 2B of the Results, we observed that the response to photoperiod was accelerated when pupae were first aged at low temperature. A series of experi-

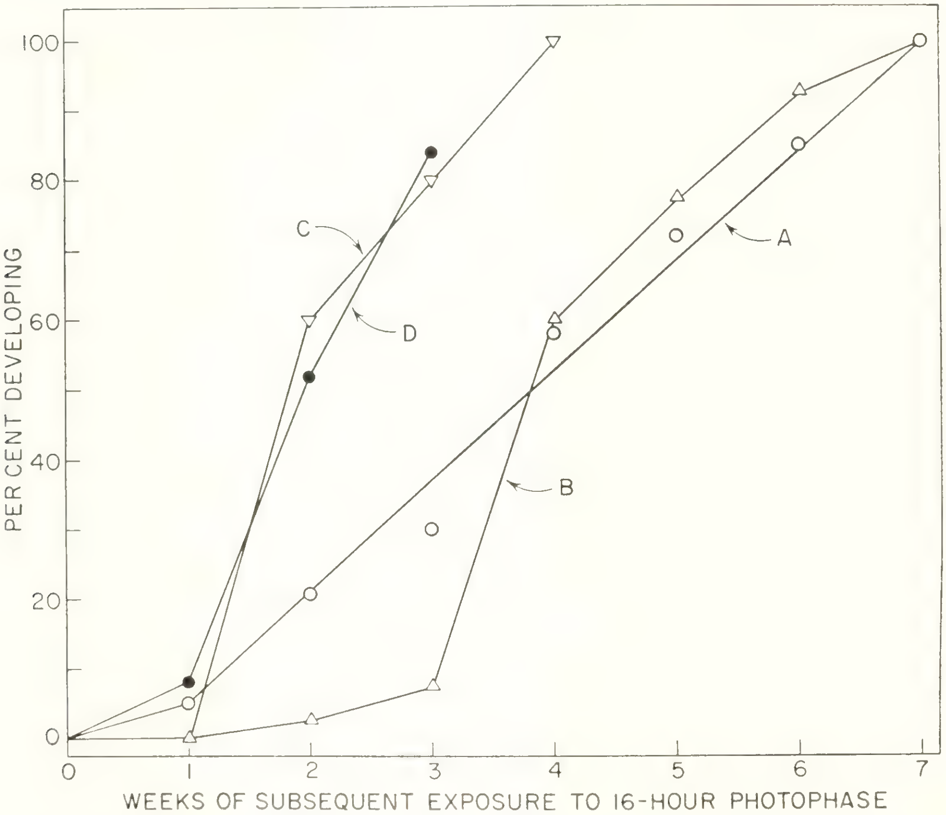


FIGURE 4. Curve A describes the termination of diapause as a function of time; 40 cocoons were incubated at 25° C. in the presence of a long day of 16 hours. In parallel experiments, recorded as Curves B, C, and D, groups of 40 cocoons were first given preliminary exposure at 25° C. to a short-day regimen for 4, 17, and 22 weeks, respectively. The curves describe the termination of diapause after return to the long-day regimen. As noted in Curve B, the preliminary 4-week exposure to the inhibitory photoperiod slowed down the subsequent response. This effect was replaced by a stimulation when the preliminary aging was prolonged to 17 weeks (Curve C) or 22 weeks (Curve D).

ments was designed to test whether the same accelerated response could be induced by aging the pupae at 25° C. in the presence of an inhibitory photophase of 8 hours. To this end, groups of 40 cocoons were first exposed to the 8-hour photophase for 0, 4, 17, and 22 weeks, respectively, and then placed at a 16-hour photophase to induce development.

As summarized (Curve B of Figure 4), the preliminary 4-week exposure to the inhibitory photoperiod slowed down the subsequent response. But when the preliminary exposure was extended to 17 (Curve C) and 22 weeks (Curve D), the inhibition was overcome. When compared to the controls (Curve A), these pupae now showed an accelerated response to the 16-hour photophase reminiscent of that seen after preliminary aging at low temperatures.

5. *Photoperiod gradients*

As described in the section on Methods, 80 unchilled pupae were exposed at 25° C. to an inhibitory 8-hour photophase at one end and a stimulatory photophase of 16 hours at the opposite end. When the experiment was terminated after seven weeks, the results were as follows (see Table II):

TABLE II

Effect of photoperiod gradients on diapausing pupae at 25°C.

Daily photophase (hrs.)		Number of animals	Cumulative % developing after (weeks)						
Head	Abdomen		1	2	3	4	5	6	7
16	8	40	0	2.5	7.5	60.0	77.5	92.5	100
8	16	40	0	0	0	0	0	0	0

All of the animals initiated development when the head-end was exposed to a daily photophase of 16 hours. None initiated development when the head-end was exposed to the inhibitory photophase of 8 hours.

This shows that the reception of the long-day stimulus occurs at the head-end of the pupa; it also shows that exposure of the abdomen to short-day conditions is ineffective in canceling-out a long-day exposure at the head-end.

6. *Transplantation of photosensitivity*

By previously described techniques (Williams, 1946, 1959), the brains were removed from 26 chilled pupae. Each brain was then reimplanted under a plastic window at the tip of the abdomen. The pupae, now with brains in their hind-ends, were positioned in the early version of the "gradient board" and the latter was placed in a 25° C. incubator programmed for an 8-hour photophase. In this manner each individual was exposed at one end to an inhibitory 8-hour photophase while the opposite end was maintained in continuous darkness. During the two-month terms of the experiment, the results were as follows:

Of the 14 individuals whose brainless anterior ends were exposed to the inhibitory 8-hour photophase, 71% initiated adult development. Of the 12 individuals whose brain-containing abdomens were exposed to the inhibitory photophase, none initiated development.

So, by the transplantation of the brain to the tip of the abdomen, the sensitivity to photoperiod was likewise transplanted.

7. *The role of the brain in the photoperiodic response*

A. *Brain removal prior to the initiation of adult development*

Brains were removed from 28 diapausing pupae and the brainless individuals were then stored at 25° C. in the presence of a 17-hour photophase. Despite exposure to this most favorable photoperiod, none of the brainless pupae underwent any development (Table III). Most individuals survived for at least six months and finally died without any trace of adult development.

TABLE III

Effects of brain removal before and after the visible initiation of adult development

Day of adult development	Number of animals	Number of moths formed	% forming moths
Prior to zero	28	0	0
0	15	3	20
1	23	6	26
2	20	17	85

This experiment demonstrates that the brain is indispensable for the initiation of adult development, and that even the most favorable photoperiod becomes completely ineffective in the absence of the brain.

B. *Brain removal after the initiation of development*

A similar group of 58 diapausing pupae was removed from cocoons and exposed to a 17-hour photophase in order to provoke the initiation of adult development. By twice-daily examinations, the zero day of adult development was identified for each individual. Brains were removed on either the zero, first, or second day of adult development and all pupae were then returned to the 17-hour photophase at 25° C. The results are summarized in Table III.

Brain removal on the zero day of adult development completely arrested the further development of 80% of individuals. The same operation performed 24 hours later blocked the further development of 74%. An additional delay of 24 hours (until the "second day of adult development") blocked the development of only 15%.

This experiment shows that in most individuals the brain completes its endocrine function about 60 hours after the visible initiation of adult development.

C. *Effects of photoperiod after the initiation of adult development*

Diapausing second-brood material was used in this experiment. One hundred previously chilled pupae were removed from cocoons, placed at 25° C., and exposed to a 16-hour photophase. On the zero day of adult development, half of the group was returned to the stimulatory 16-hour photophase; the other half was transferred to an inhibitory 8-hour photophase. Both groups emerged as adult moths after an average of 17 days.

Manifestly, photoperiod loses all its influence on the pace of adult development after the latter has actually begun. The formation of the moth then proceeds at a rate dictated by environmental temperature and without any further reference to photoperiod.

Discussion

1. *The induction and termination of diapause in A. pernyi*

As mentioned in the introduction, the detailed studies of Tanaka (1950a, 1950b, 1950c; 1951a, 1951b) have already demonstrated that the induction of diapause in the Pernyi silkworm is controlled by photoperiod. Tanaka's data on the induction of diapause by photophases within the physiological range of 8 to 18 hours are summarized as the hatched line in Figure 5. For comparison, we record as the unbroken line our data for the photoperiodic control of the termination of pupal diapause in previously chilled Pernyi. It is clear that those photoperiods which are effective in inducing diapause are also effective in stabilizing diapause once the latter has begun. Moreover, the photoperiods which are effective in preventing the onset of diapause are precisely the same as those which provoke the termination of diapause.

The fit of the two sets of data is remarkable if one considers that the investigations were performed independently fifteen years apart. In this connection we may note the strategic position of the photophase of 14 hours as the transition between "short-day" and "long-day" conditions.

The obvious inference is that the same photoperiodic mechanism which controls the induction of diapause is retained by the pupa to control the termination of diapause.

2. *The role of the brain in the induction of diapause*

In the Cecropia silkworm the brain is known to play a key role in the induction of pupal diapause. The dormant condition is, in fact, a syndrome of endocrine deficiency due to a failure of the brain to secrete a hormone prerequisite for the initiation of adult development (Williams, 1946, 1952, 1956). This failure is attributable, in turn, to an inactivation of the brain during the prepupal period (Williams, 1952). By contrast, in non-diapausing pupae of *Actias selene*, *Actias luna,* and *Antheraea pernyi*, the pupal brain is not turned off but retains its full activity. Consequently, within a few days after pupation, sufficient brain hormone is secreted to cause the initiation of adult development. If the brain is excised before this activation is complete, then the potentially non-diapausing pupae are forced to diapause (Williams, 1952; Shappirio and Williams, 1957).

Manifestly, the decision to diapause or not to diapause is dictated by what happens to the brain's endocrine activity during pupation. And in the case of *A. pernyi,* as we have seen, what happens to the brain's activity is conditioned by the photoperiods experienced during larval life.

It is important to note that a photoperiod which induces diapause does not immediately shut-off the larval brain. If it did so, pupation would be blocked and

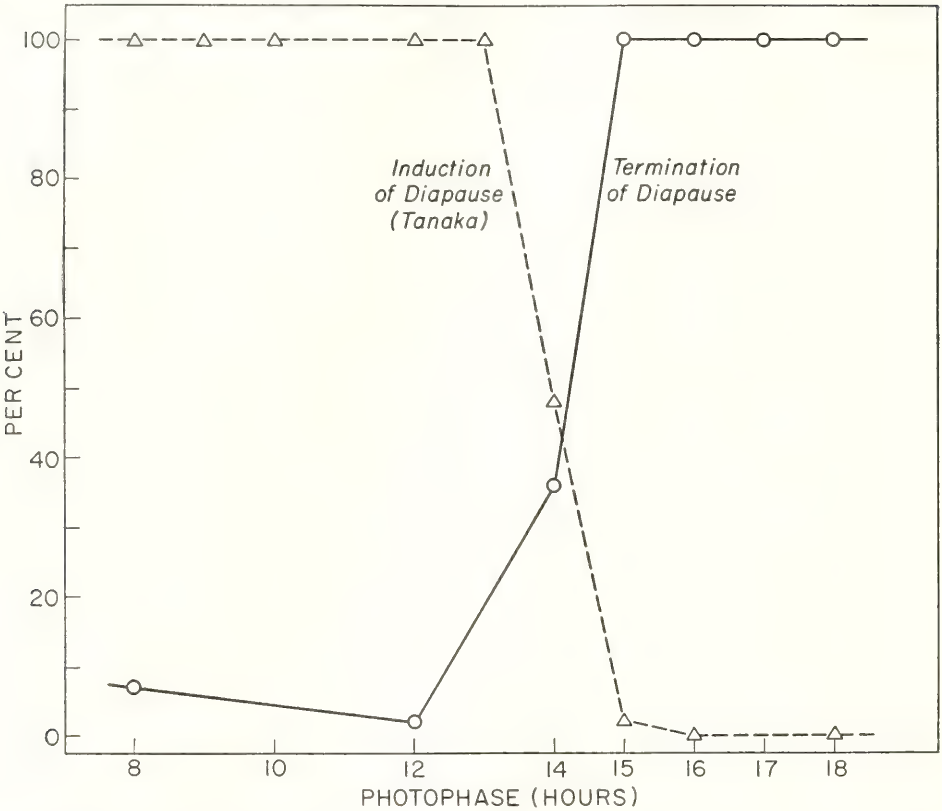


FIGURE 5. The solid line records the effects of photoperiod on the termination of pupal diapause by previously chilled *A. pernyi.* The hatched line shows the influence of photoperiod on the induction of diapause (data of Tanaka). The same short-day conditions which induce pupal diapause are also effective in stabilizing diapause. Moreover, the long-day conditions which prevent pupal diapause are the same as those which cause it to terminate.

one would observe a larval rather than a pupal diapause. The action of photoperiod is to program either the shut-down or the sustained activity of the brain after the latter has secreted sufficient hormone to cause pupation. This state-of-affairs points to some unknown mechanism for the integration and latent storage of daily photoperiod signals accumulated during larval life.

3. *The role of the brain in the termination of diapause by photoperiod*

The results of the present study show that photoperiod is ineffective when it acts on brainless pupae. Even a 17-hour photophase is then incapable of causing the termination of diapause. So, by excising the brain, one effectively removes the vehicle for the photoperiod response.

This conclusion is further affirmed by the experiments in which the brain was removed after the initiation of adult development. During the first 60 hours of adult development, the brain hormone completes its tropic action on the prothoracic glands and the brain, itself, is no longer necessary for the continuation of adult development. By this time, photoperiod has become inconsequential and incapable of influencing the further course of events.

We are therefore persuaded that the photoperiod presides over both the onset and the termination of pupal diapause by controlling the endocrine function of the brain.

4. *Direct or indirect action of photoperiod on the brain?*

In a brief report on the photoperiod response of *A. pernyi,* Shakhbazov (1961) called attention to the transparent facial cuticle which overlies the pupal brain. When this zone was coated with black paint, the pupae behaved as if they were in continuous darkness. Shakhbazov concluded that light is transmitted through both the cocoon and the facial "window" to act on some organ in the pupal head.

In the present study this conclusion has been further documented by exposing individual pupae to photoperiod gradients. Long-day conditions promoted the termination of diapause only when they acted on the head-end. In like manner, short-day conditions were effective in maintaining diapause only when they acted on the head-end. By contrast, exposure of the abdomen to either long- or short-day conditions was without any detectable effects.

The cephalic action of photoperiod has been previously described by Lees (1960) in studies of the aphid, *Megoura.* These remarkable experiments have now been extended and reported in full detail (1964). By pin-pointing tiny beams of light through hollow needles or plastic filaments, Lees was able to show that the photosensitivity of the aphid is confined to the head and that the central region of the head is particularly important as a light pathway.

The present study is in full agreement with Lees' findings and provides the first direct evidence that photoperiod acts on the brain itself. Thus, when the brain was excised from the head and reimplanted into the tip of the abdomen, the entire mechanism of the photoperiod response was thereby transplanted to the hind-end. So, in the case of *A. pernyi* the evidence is little short of conclusive that photoperiod acts directly on the brain.

This conclusion is contrary to that which Beck and Alexander (1964a, 1964b) have recently proposed on the basis of their studies of the termination of larval diapause in the European corn-borer, *Ostrinia nubilalis.* In this species, photoperiod is reported to act on certain cells in the mucosa of the anterior region of the hindgut. These cells are said to secrete a brain-stimulating hormone ("proctodone") which under long-day conditions is in phase with a circadian rhythm of brain reactivity; under short-day conditions, proctodone is ineffective because it is secreted out-of-phase with the endogenous brain rhythm. The new hormone is alleged to play a key role in embryonic and postembryonic development, as well as "in non-diapause growth, polymorphism, periodism, and the several forms of diapause" (Beck and Alexander, 1964b).

It is not our present purpose to discuss the new theory in detail. Proctodone has not entered into our calculations for in *A. pernyi,* as we have seen, it is the head-end which is sensitive to photoperiod and, within the head-end, the brain itself. In *A. pernyi,* we have found no trace of the mechanism described by Beck and Alexander.

5. *The endocrine mechanism*

The present study provides the first experimental proof that the photoperiod acts directly on the brain, itself, to control and modulate the secretion of brain hormone. As mentioned above, this conclusion has long been implicit in the pioneering studies of Lees (1955, 1960, 1964) on the photoperiodic responses of aphids.

We shall postpone to a later occasion detailed consideration of how photoperiod acts on the brain to control the secretion of brain hormone. For present purposes, suffice it to say that the minimal brain mechanism presumably includes the following: (1) a pigment for the absorption of appropriate wave-lengths of light; (2) a timing mechanism which counts the hours of darkness (and perhaps also the hours of light); (3) an output from the clockwork-computer to the neurosecretory cells of the brain; and (4) some sort of physiological "needle-valve" for regulating the secretion and translocation of brain hormone.

SUMMARY

1. In the oak silkworm, *Antheraea pernyi,* photoperiod controls not only the onset of pupal diapause (as previously demonstrated by Tanaka) but also the termination of pupal diapause.

2. At 25° C., short-day conditions (4- to 12-hour photophases) strongly inhibit the termination of pupal diapause; maximal inhibition is by a 12-hour photophase.

3. Long-day conditions (15- to 18-hour photophases) promote the termination of diapause; a 17-hour photophase is the most effective.

4. A 14-hour photophase is transitional between short-days which sustain diapause and long-days which terminate diapause.

5. By various experimental maneuvers, sensitivity to photoperiod was localized in the head-end of the pupa. Short-day illumination of the head-end inhibited the termination of diapause even when the hind-end was exposed to long-day conditions. In like manner, long-day illumination of the head-end was fully effective even when the abdomen received short-day illumination.

6. When the brain was removed from the head and implanted into the tip of the abdomen, the sensitivity to photoperiod was thereby shifted to the hind-end.

7. Additional experiments indicated that the brain is the vehicle for the reception and implementation of photoperiod signals. Brainless pupae are insensitive to photoperiod, while normal pupae are sensitive to photoperiod during the period when development is dependent on the secretion of brain hormone. When this period terminates about 60 hours after the initiation of adult development, photoperiod has lost all its effectiveness.

8. It is concluded that photoperiod acts directly on the brain, itself, to modulate the secretion of brain hormone and thereby to control the termination of pupal diapause.

NOTE ADDED TO PROOF

Since this manuscript was submitted, additional studies have demonstrated that diapausing pupae of *Antheraea polyphemus* respond to photoperiod in essentially the same manner as here described for *A. pernyi*. The same appears to be true for diapausing (unchilled) pupae of *Hyalophora cecropia*.

LITERATURE CITED

Adkisson, P. L., 1964. Action of the photoperiod in controlling insect diapause. *Amer. Nat.* (in press).

Beck, S. D., and N. Alexander, 1964a. Hormonal activation of the insect brain. *Science,* **143:** 478–479.

Beck, S. D., and N. Alexander, 1964b. Proctodone, an insect developmental hormone. *Biol. Bull.,* **126:** 185–198.

Dutky, S. R., M. S. Schechter and W. R. Sullivan, 1962. A lard-can device for experiments in photoperiodism. *J. Econ. Entomol.,* **55:** 575.

Harvey, W. R., and C. M. Williams, 1958. Physiology of insect diapause. XII. The mechanism of carbon monoxide-sensitivity and -insensitivity during the pupal diapause of the Cecropia silkworm. *Biol. Bull.,* **114:** 36–53.

Lees, A. D., 1955. The Physiology of Diapause in Arthropods. Cambridge University Press.

Lees, A. D., 1960. Some aspects of animal photoperiodism. *Cold Spr. Harb. Symp. Quant. Biol.,* **25:** 261–268.

Lees, A. D., 1964. The location of the photoperiodic receptors in the aphid *Megoura viciae* Buckton. *J. Exp. Biol.,* **41:** 119–133.

Shakhbazov, V. G., 1961. The reaction of the length of daylight and the light receptor of the pupa of the Chinese oak silkworm *Antheraea pernyi G. Dok. Akad. Nauk* SSSR, **140,** No. 1: (AIBS) 944–946.

Shappirio, D. G., and C. M. Williams, 1957. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Royal Soc. London, Ser. B,* **147:** 233–246.

Tanaka, Y., 1950a. Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar-silkworm. I. *J. Seric. Sci. Japan,* **19:** 358–371. (*In Japanese*).

Tanaka, Y., 1950b. Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar-silkworm. II. *J. Seric. Sci. Japan,* **19:** 429–446. (*In Japanese*).

Tanaka, Y., 1950c. Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar-silkworm. III. *J. Seric. Sci. Japan,* **19:** 580–590. (*In Japanese*).

Tanaka, Y., 1951a. Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar-silkworm. V. *J. Seric. Sci. Japan,* **20:** 132–138. (*In Japanese*).

Tanaka, Y., 1951b. Studies on hibernation with special reference to photoperiodicity and breeding of the Chinese Tussar-silkworm. VI. *J. Seric. Sci. Japan,* **20:** 191–201. (*In Japanese*).

de Wilde, J., 1962. Photoperiodism in insects and mites. *Ann. Rev. Entomol.,* **7:** 1–26.

Williams, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm *Platysamia Cecropia. Biol. Bull.,* **90:** 234–243.

Williams, C. M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.,* **103:** 120–138.

Williams, C. M., 1956. Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.,* **110:** 201–218.

Williams, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull.,* **116:** 323–338.

Williams, C. M., 1963. Control of pupal diapause by the direct action of light on the insect brain. *Science,* **140:** 386.

Williams, C. M., and P. L. Adkisson, 1964. Photoperiodic control of pupal diapause in the silkworm, *Antheraea pernyi. Science,* **144:** 569.

STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES. V. ACCELERATION OF RECOVERY OF PHOSPHORYLATION BY POLYANIONS[1]

HENRY T. YOST, JR., STEWART S. RICHMOND AND LAURENCE H. BECK

Department of Biology, Amherst College, Amherst, Mass.

While the major part of the effect of cell-free extracts of spleen on the recovery of irradiated animals may be attributed to cellular contamination (Koller, Davies and Doak, 1961), there are several studies which suggest that such extracts may be effective in accelerating the recovery of irradiated animals (Ellinger, 1956, 1957; Panjevac, Ristic and Kanazir, 1958). It must be clear that, regardless of the source of the cells, regeneration of radiation-damaged areas involves a growth process for which energy must be supplied. Since the great bulk of energy available for growth in higher organisms is supplied by the process of oxidative phosphorylation, and since it has been demonstrated that oxidative phosphorylation is uncoupled largely as an indirect effect of exposure to radiation (Benjamin and Yost, 1960; Yost, Glickman and Beck, 1964), it seemed logical to investigate the effects of various protective agents on the phosphorylating mechanism. The common characteristic of cell-free extracts is that the effective agent appears to be a compound of high molecular weight, probably a nucleic acid or nucleoprotein (Cole and Ellis, 1954). The finding of Allfrey and Mirsky (1958) that polyanions are capable of restoring phosphorylative activity in isolated nuclei suggested the possibility of a dependence upon polyanions of all phosphorylating mechanisms. Consequently, tests of various polyelectrolytes were designed to see what effect their injection into irradiated animals might have.

Since preliminary experiments indicated that the phosphorylating mechanism was protected by cell-free spleen extract, it seemed possible to extend the investigation to a consideration of the relationship of oxidative phosphorylation to recovery processes in general. If the process of oxidative phosphorylation be central to the regeneration process, restoration of phosphorylation at an appropriate time should make possible higher survival in irradiated organisms. Therefore, a secondary consequence of the initial investigation was the testing of some of these compounds for their ability to promote survival in irradiated animals, both as a study of the general problem of protection against radiation damage and as a test of the hypothesis that damage to the phosphorylating mechanism is causally related to the recovery process.

MATERIALS AND METHODS

All experiments were carried out with male, albino rats of the Sprague-Dawley strain, weighing between 125 and 175 grams. The rats were irradiated without

---

[1] This work was supported in part by a grant (CA-06132) from the National Cancer Institute.

anesthetic in a small wire cage which permitted little freedom of motion. All radiation was delivered from a 175-curie Co^{60} source, filtered with one-half inch of Lucite, at an intensity of 65 r/min. All rats received 800 r total-body, the dose being calculated for a plane passing through the pituitary and spleen of the animal.

Rats were sacrificed by a sharp blow to the head, and the tissue for study was removed immediately. In the case of spleen tissue, two or three spleens were pooled and homogenized; for liver, a slice weighing approximately 2½ grams was removed from each rat; and in the case of the testis, four testes (two rats) were utilized for a single assay. The tissue was homogenized in cold isotonic sucrose containing 0.005 *M* disodium versenate. The mitochondria were isolated from the homogenate by differential centrifugation (Schneider, 1948), with the particulates collected at 9000 *g* and washed once. Mitochondria from control spleens and from liver, whether experimental or control, were resuspended in one ml. of sucrose solution. Mitochondria from irradiated spleen and from testis were resuspended in 0.5 ml. sucrose solution. One-half ml. of mitochondrial suspension was added to each Warburg flask for assay. The unequal dilutions of the different preparations were made necessary by the differences in yield of mitochrondria from the various organs and animals. These dilutions provide a method for getting reproducible results and provide the most conservative way of obtaining these results (Yost, Glickman and Beck, 1964).

The efficiency of respiratory energy conversion was measured using the P:O ratio. Oxygen uptake was measured in a Warburg respirometer at 25° C. Readings were taken for 20 to 30 minutes, after a five-minute equilibration period. Satisfactory P:O ratios were obtained from concentrated preparations, so long as 6 micro-atoms of oxygen were consumed within that period. Succinate was used as the substrate, and the incubation medium was the same as that described previously (Yost and Robson, 1959). Estimation of the remaining inorganic phosphorus was carried out by the method of Lowry and Lopez (Glick, 1949). All phosphate tests were run in duplicate.

For the preparation of the cell-free spleen extract, a slight modification of the procedure of Ellinger (1956) was employed. Sampling of spleens from animals weighing the same as those to be injected indicated an average spleen weight of 0.80 gram. Since it was desired to inject each rat with 2.5 ml. of extract containing the material from one spleen, the entire collection of removed spleens was weighed and then homogenized in a volume of cold 0.9% NaCl solution equivalent to 2.5 ml. per spleen. The homogenate was centrifuged for 30 minutes at 600 *g* to remove gross particulate matter (primarily nuclei and cell fragments). The supernatant fraction was filtered through several layers of filter paper in a Buechner funnel and then, under high vacuum, through a Selas bacteriological (0.3) filter. From this point on, sterile technique was followed. The resulting extract was transferred to vaccine bottles and kept cold until use (extracts were kept no longer than two days from the time of preparation). Samples were examined under the microscope for whole-cell counts. In all cases the extract was found to be devoid of whole spleen cells and of bacterial contamination. (As an added check, spleen preparations were spread on nutrient agar but no colony growth was observed.) Appropriate aliquots were withdrawn and injected intraperitoneally.

In the preparation of the extracts used to test the activity of various fractions of the homogenate, nuclei were separated from the rest of the material by centrifugation at 600 *g*. Prior to centrifugation the homogenized spleens were filtered through four layers of fine cheesecloth to remove whole cells and much of the cellular debris. This technique permitted the passage of many nuclei, which could be observed in the microscope. Precipitated nuclei were washed by resuspending in sucrose and recentrifugation. After washing, the resuspended nuclear pellet was allowed to stand for 30 minutes, during which time the nuclei settled to the bottom. The top 95% of the liquid was withdrawn and the remaining suspension was used to prepare an extract (by steps similar to those above) labeled the "nuclear" fraction. The supernatant fraction was derived from the several centrifugations made during the isolation of the nuclei. This collected supernatant was centrifuged at speeds slightly in excess of 600 *g*. The supernatant was decanted and then repeated centrifugations were made until no sediment was detected. The resulting clear, deep red solution was used as the "supernatant fraction." From such a procedure it is clear that the nuclear fraction is derived primarily from cell nuclei, although a certain amount of cytoplasmic material may be carried along. In the case of the supernatant fraction, it is clear that the extract contains a large number of elements other than nuclei, including the mitochondria, etc. The sum of the two fractions would not contain all of the elements to be found in an extract of a complete homogenate of cells. In the repeated washings and centrifugations, a number of elements are lost. Thus, three rather different extracts were made: one from whole-cell homogenates, one from homogenates of isolated nuclei, and one from "homogenates" of cellular materials smaller than those precipitated in the nuclear fraction. These three extracts are identified in Table I as "whole homogenate," "nuclear fraction" and "supernatant."

TABLE I

Effects of various cell-free spleen extracts on oxidative phosphorylation in spleen mitochondria from rats receiving 800 r total-body

Days after exposure	Non-injected	Saline	Whole homogenate	Supernatant	"Nuclear" fraction	No. runs
1	1.4	1.3	1.8	1.1	1.8	5
3	1.5	1.5	1.9	1.2	1.8	5
5	1.4	1.4	1.9	1.2	1.9	3
8	1.4	1.4	1.9	1.1	1.8	3
Control (ave.)	1.9	1.9	1.9	1.9	1.9	16

Three classes of compounds were tested for their effect upon the phosphorylation mechanism: spleen extract, polycations and polyanions. Rats receiving the spleen extract were given injections of 2.5 ml.; in each case one set of control rats were injected with 2.5 ml. of 0.9% NaCl. The other compounds were injected in volumes ranging from 0.5 to 2.0 ml. In these latter cases, both irradiated and unirradiated control rats were injected with an equal volume of saline or water, depending upon the method of preparation of the experimental compounds. The polyanions used were polyethylene sulfonate, 5900 and 12,500 M.W. (Upjohn

Co.), desoxyribonucleic acid, standard and high polymer (Nutritional), and ribosenucleic acid, yeast (Nutritional). The polycations used were salmine (Nutritional) and lysozyme (Worthington). The lysozyme was inactivated by heat prior to injection. All injections were made intraperitoneally, in the anterior-abdominal region.

Survival tests were run for 30-day periods. In each test 90 rats were used, divided as follows: controls, 800 r whole-body, and 800 r whole-body injected with polyethylene sulfonate. Daily counts of the rats were kept and all tests were repeated three times.

RESULTS

The design of the experiments and the method of presenting the data have been discussed previously in detail (Yost, Glickman and Beck, 1964). The following two paragraphs are intended to summarize the previous discussion and to facilitate interpretation of the data.

The data in the various tables are presented as average P:O ratio values for each set of experiments done at a particular condition, since the number of experiments reported in this paper is too great to permit the presentation of each experiment as a separate datum. In the case of the controls, the values for all runs have been averaged, since the variation for control animals is relatively small and repeated assays of this type have yielded control average values of 1.8 or 1.9 for livers and spleens, and 1.2 for testes. There is, of course, a certain amount of variation intrinsic to the P:O ratio determinations. Thus, one might expect, for an average P:O value of 1.9, to find a range of values in a set of individual experiments running from 1.7 to 2.2, or, for a value of 1.4, a range of values from 0.9 to 1.6. In each case, however, a clear difference exists between experimental and control animals. There is *never* an overlap of the values in individual experiments, so long as inactivation occurs. The consistency of these findings is remarkable, considering the internal variations to be expected in the system, and this suggests that it is perfectly permissible to present only the average values without presenting the range of each mean value. Percentage inactivations can be derived from the control values in each column of the data tables. As a check for the validity of this method, calculations were made on the basis of the average data and on the basis of the individual runs; in all cases the values were essentially the same. Thus, there is no distortion of the meaning of the data by the method of presentation.

Similarly, there is difficulty in the development of a test for the significance of differences in data of this type. Where the number of tests is sufficiently great (6 to 10 runs), standard deviation values of approximately 0.15 are obtained. Thus, to get highly significant results, it is necessary that the differences in P:O ratios between controls and experimentals be greater than 0.5. In Table I (and other tables as well) some lines of data can be demonstrated to be significantly different from one another on the basis of standard deviation tests, but most of the lines cannot. On the other hand, if the data for different days are summed and averaged, there are highly significant differences on the basis of standard deviation analysis. Furthermore, since the pattern remains unchanged and since experimental values at no time overlap control values (so long as an inactivation has occurred), it seemed permissible to lump the data for statistical treatment by Chi-square analysis. When this is done the difference between the experimentals and their appropriate controls is highly significant ($P < 0.001$). Thus, the data in this paper clearly indicate that inactivation of oxidative phosphorylation occurs after exposure to radiation, and that the administration of several different compounds is capable of either removing or preventing this inactivation.

The data in Table I show that a cell-free extract of spleen tissue is capable of restoring oxidative phosphorylation in irradiated rats. The rats were injected shortly after exposure and assayed at various times after exposure. It can be seen that the "protective" effect of the extract is observable 24 hours after exposure. At this time, the effect of the irradiation is at its maximum in the control-irradiated animals (Yost, Glickman and Beck, 1964). Furthermore, the effect of the extract appears to be permanent; that is, it does not recede with time. Thus, it appears that cell-free spleen extracts are capable of making a permanent restoration of the phosphorylating mechanism in the surviving cells of the spleen. Furthermore, it is clear that the activity of this extract is limited to the nuclear fraction of the homogenate. Thus, whatever the protective agent may be, it appears to be localized in the nuclei of the spleen cells from which the extract is made. Neither saline nor saline extract of the non-nuclear fraction (designated "supernatant" in Table I) is capable of restoring the phosphorylating mechanism.

In an attempt to assess the general nature of the protective effect demonstrated by the data presented above, rats were injected with a cell-free spleen extract of the whole homogenate type immdiately after irradiation, and the phosphorylating ability of liver and testis mitochondria was assayed at various times after exposure (Table II). In liver and testis, maximum damage has been achieved by 48 hours after exposure, and therefore, two-day intervals were taken as a test of the regeneration of the system. Since after 10 days liver phosphorylation has recovered without treatment, only the first 8 days post-irradiation were used for assay. In the case of the testis, assays were made until the 14th day, after which period normal recovery has been effected. It can be seen that the extract is capable of restoring the phosphorylating mechanism on a permanent basis in both the liver and testis mitochondria. Therefore, the effect of the spleen extract is not specifically upon spleen cells; rather, it is a general effect of some component derived from the spleen cells. In both cases, there was no significant effect of the saline extraction medium on the recovery of the phosphorylating mechanism.

On the basis of the observations of Allfrey and Mirsky (1958) that polyanions restore uncoupled nuclear phosphorylation, it was decided to try a synthetic polyanion to see if it would replace the spleen extract in the recovery process. The data presented in Table III indicate that polyethylene sulfonate is just as effective as the spleen extract in causing the rapid and permanent recovery of the phosphorylating mechanism in irradiated rats. In this case, as in the case of the spleen extract, injections of polyethylene sulfonate were made immediately following irradiation, and assays were made at various times after exposure. The effect of the polyethylene sulfonate has been achieved by 24 hours after exposure and continues throughout the assay period. Furthermore, both the 12,500 MW

TABLE II

Effect of cell-free spleen extract on oxidative phosphorylation in liver and testis mitochondria from rats receiving 800 r total-body

Days after exposure	Liver			Testis		
	Sham-injected	Injected	No. runs	Sham-injected	Injected	No. runs
2	1.2	1.8	6	0.8	1.2	6
4	1.3	1.8	6	0.8	1.1	6
6	1.5	1.8	6	0.9	1.2	6
8	1.5	1.8	6	—	—	—
10	—	—	—	0.8	1.2	6
14	—	—	—	1.0	1.2	6
Control (ave.)	1.8	1.8	24	1.2	1.2	30

and 5900 MW compounds are satisfactory for restoring the phosphorylating mechanism. Thus, a molecular weight of no more than 6000 is necessary to effect the observed change. To demonstrate the significance of the protective effect of PES, the data from Table III were used to make a Chi-square analysis of the null hypothesis that there would be no significant difference in the distribution of either the sham-injected or injected values above or below the minimum normal value of 1.8. The Chi-square values indicate that the distribution of the sham-injected values below 1.8 and the injected values above 1.8 is highly significant, with the probability of occurrence by chance less than 0.001. This rejection of the null hypothesis clearly shows that PES is responsible for the normal values obtained in the experimental rats.

Having demonstrated that the polyanion, PES, was capable of restoring oxidative phosphorylation in irradiated rats, it seemed logical to test different polyanions and polycations. Table IV presents the data obtained with three polyanions (PES, RNA and DNA) and two polycations (salmine and lysozyme). The assays were made on liver mitochondria from a single rat liver, to avoid

TABLE III

Effect of polyethylene sulfonate on phosphorylation in spleen mitochondria from rats receiving 800 r total-body

Days after exposure	12,500 MW			5900 MW		
	Sham-injected	Injected	No. runs	Sham-injected	Injected	No. runs
1	1.6	1.9	5	1.4	1.9	5
2	1.5	1.9	3	—	—	—
3	1.4	1.9	3		—	—
8	1.5	1.9	5	1.4	1.8	5
14	1.5	1.9	7	—	—	—
Control (ave.)	1.9	1.9	23	1.4	1.9	10

pooling two or three rats as is necessary in analyses with spleen mitochondrial preparations. It can be seen that PES restores the liver phosphorylation, as it did the spleen phosphorylation, and thus the system seems adequate for comparisons of other polyanions and polycations. The data indicate that polyanions restore oxidative phosphorylation and that polycations are incapable of making the restoration. In the case of highly polymerized DNA (DNA-2), no effect on phosphorylation was observed. Unfortunately, with highly polymerized DNA preparations, it is hard to be sure that the injection is effective since the viscosity of the solution makes intraperitoneal injections extremely difficult. Furthermore, when the molecular weight is raised to a very high level, it may be possible that permeability problems become overpowering. This might be the reason for the failure for the polycation, lysozyme, to have any effect on the phosphorylating mechanism. On the other hand, it seems unlikely that salmine would be incapable of penetrating, and, therefore, these data can be taken as presumptive evidence that the polycations have a different effect than the polyanions on the phosphorylating mechanism of irradiated rats. It is certainly clear that lower

TABLE IV

Effect of various polyelectrolytes on oxidative phosphorylation in liver mitochondria from rats receiving 800 r total-body

	12 hours post-irrad.	2 days post-irrad.	8 days post-irrad.
Sham-injected	1.5	1.6	1.6
PES	2.0	1.9	1.9
No. runs	6	6	4
Sham-injected	—	1.5	1.5
RNA	—	1.9	1.8
No. runs	—	10	3
Sham-injected	1.3	1.5	1.5
DNA-1*	1.8	1.9	1.9
No. runs	3	5	3
Sham-injected	—	1.6	—
DNA-2*	—	1.6	—
No. runs	—	4	—
Sham-injected	1.2	1.2	1.4
Salmine	1.2	1.2	1.4
No. runs	3	4	3
Sham-injected	—	1.2	—
Lysozyme	—	1.3	—
No. runs	—	3	—
Controls (ave.)	1.9	1.9	1.8
No. runs	12	21	13

* DNA-1 is the usual commercial grade; DNA-2 is highly polymerized.

TABLE V

Effect of polyethylene sulfonate, injected pre- and post-exposure to 800 r total-body, on spleen phosphorylation

Days after exposure	24 hours post-ex.			5 hours pre-ex.		
	Sham-injected	Injected	No. runs	Sham-injected	Injected	No. runs
2	1.6	2.0	5	1.4	1.5	3
8	1.5	1.8	4	1.5	1.5	3
Control (ave.)	1.9	1.9	9	1.9	1.9	6

molecular weight polyanions, such as RNA, DNA and PES, are capable of restoring the phosphorylating mechanism on a permanent basis.

The data in Table V show the effect of polyethylene sulfonate when injected either prior to or after exposure to radiation. Although results with spleen are presented here, similar experiments have been done with liver mitochondrial preparations and the data are essentially the same. The most striking aspect of these results is that PES can be injected 24 hours after the exposure to radiation and still result in a complete recovery of the phosphorylating mechanism. On the other hand, pre-irradiation injection of PES appears to have no effect. Thus, in the five hours prior to radiation, either the PES is incorporated (or excreted) by the rat and is no longer available to do whatever it does in the restoration process, or the exposure to radiation alters the PES in such a way that it is no longer effective.

Having demonstrated that the PES can restore the phosphorylating mechanism, it seemed wise to test the effect of this compound upon survival. Table VI presents the data derived from survival studies performed under different conditions: two different doses of radiation, and two methods of injection of the PES. It can be seen that there is no significant difference in the survival of animals injected

TABLE VI

Survival of rats receiving total-body irradiation and polyethylene sulfonate

Treatment	Initial	Final	% Survival
Control	120	120	100
400 r	120	98	82
400 r + PES*	120	97	81
Control	120	118	98
800 r	120	38	32
800 r + PES*	123	38	31
Control	120	119	99
800 r	120	43	36
800 r + PES**	120	50	42

* 20 mg. in 2 ml.

** 20 mg. in 0.5 ml.

with PES over those which were uninjected, and, thus, it would appear that PES is capable of influencing the regeneration of the phosphorylating mechanism without influencing survival.

DISCUSSION

The results presented in this paper show that a non-biological polyanion, PES, is capable of restoring oxidative phosphorylation in irradiated rats and that the effect of this compound is essentially the same as that of RNA, DNA or cell-free spleen extracts. Thus, the results obtained here are quite similar to those obtained by Allfrey and Mirsky (1958) with nuclear phosphorylation, indicating that either a polymer of DNA or a similarly charged polymer (PES) could restore an uncoupled phosphorylating mechanism. Apparently the action of PES in irradiated rats is the same as, or at least very similar to, the action of the nucleoprotein extracted from spleen and used by Cole and Ellis (1954) and by Ellinger (1957).

Since the PES can be injected as much as 24 hours after exposure to radiation and still restore the mechanism, it is clear that the polyanions are not acting as protective agents in the usual sense. That is to say, their function seems to be "restorative," agents which act on the regeneration process. Since at 24 hours both the phosphorylating mechanism and the lymphocyte population of the spleen are at their lowest levels (Yost, Glickman and Beck, 1964), it is clear that the radiation damage, through whatever channel it may be effected, has already been achieved. From this point on, only the regeneration mechanism can be affected by the various agents. Thus, the effect of polyanions must be attributed to their ability to restore the phosphorylating chain in some way, rather than any effect they might have in removing "toxic substances" produced by the radiation.

The function of polyanions in restoring the phosphorylation process is obscure; however, a certain parallel may be drawn betwen the studies presented here and other work on the normal mechanism of coupling in isolated phosphorylating particles. It has been demonstrated several times that mitochondrial RNA has a role in the normal coupling of phosphorylation and oxidation (Yost and Robson, 1959; Hanson, 1959; Linnane and Titchener, 1960). Pinchot (1959) has shown that an RNA "cofactor" is required to bind a soluble, heat-labile fraction essential for phosphorylation to the cytochrome fraction serving as an electron transport system. The binding is nonspecific, since it is possible to replace the extracted "cofactor" with synthetic polynucleotides. Thus, it would appear that the function of a polyanion might be the binding of phosphorylating enzymes to the electron transport site (most likely, by a ligand with the aid of Mg^{++}).

If the preceding hypothesis for the function of polyanions be correct, the effect of the polycations might only be detected at later dates. If the polycations were incorporated into the system in place of polyanions, they might stabilize the uncoupling by preventing the entrance of new RNA polymers to the phosphorylating site and thus prevent "wound healing." An examination of the long-term effect of polycations on uncoupled preparations is in process, but the data do not permit any conclusions at the present time.

It is possible that the role of the polyanion is the removal of a naturally-occurring uncoupling agent released from damaged mitochondria, since it has been demonstrated that the process of ageing results in structural changes in the mitochondria which release an uncoupler composed primarily of lipids and hemeproteins (frequently called mitochrome). This seems quite possible in view of the finding of Lehninger (1959) that the lipid uncoupling agents are probably released under the influence of hormones (such as thyroxin) and the finding of Wojtczak and Wojtczak (1960) that serum albumen protects mitochondrial phosphorylation by complexing with fatty acids. If such a mechanism were the correct explanation of the effect of the polyanions, it would be possible to duplicate the effect with *in vitro* experiments. Experiments were performed in which either DNA-1 or PES were added to isolated mitochondria. In neither case was it possible to obtain a restoration of the phosphorylating mechanism. In the case of PES, 20 mg. and 40 mg. were added to each Warburg flask, but no effect of the polyanion could be demonstrated. Thus, the function of the PES is not through the removal of an uncoupling agent in the mitochondrial preparation. Apparently it is necessary for the intact cell to aid in the incorporation of the polyanion to achieve the recoupling of phosphorylation.

The failure of PES to increase survival may be interpreted in three ways. In the first place, it may be taken as a direct confirmation of the cellular repopulation hypothesis, suggesting that nothing but the addition of cellular elements will increase the regeneration process in irradiated organisms (Koller, Davies, and Doak, 1961). Second, it may be taken to indicate that, in those organisms which are destined to survive in the population, enhancement of the recovery process by restoration of oxidative phosphorylation will only result in an increased *rate* of recovery and not in a significantly increased number of survivors. Unfortunately, the way the survival studies are generally conducted does not test this hypothesis. It is necessary to study the day-by-day events at a histological level to determine whether or not the injection of PES is capable of accelerating the rate of recovery. Such studies are now in progress. Finally, the data may indicate that the uncoupling of phosphorylation is only indirectly related to the regenerative process. The uncoupling may be a generalized stress response of the rat which has the net effect of accelerating the general metabolism to permit a more rapid synthesis of intermediate compounds necessary to "rebuild" damaged areas.

It may well be that if proper concentrations of PES are tried, increases in survival will be found. It must be remembered that certain cell-free spleen extracts increase both the regeneration of the phosphorylating mechanism (these data) and survival (Ellinger, 1957). Therefore, it is possible that the failure of the PES to increase survival is a concentration problem (or a molecular weight problem). Thus, before any final conclusions can be drawn about the value of polyanions in survival studies, it will be necessary to carry out extensive experiments on the survival of treated organisms. Such studies are beyond the scope of this laboratory.

The authors are indebted to Dr. Hope H. Robson, Martha T. Yost and Ralph E. Barrett for assistance in the course of the work and in the preparation of the manuscript. The authors are very much indebted to Dr. Merrill E. Speeter of the Upjohn Company, who made the polyethylene sulfonate available for these studies.

SUMMARY

Male rats exposed to 800 r gamma radiation were injected post-irradiation with various polyanions and polycations and with a cell-free extract of rat spleen. Inactivation of oxidative phosphorylation by the irradiation was reversed by the spleen extract and by the polyanions, but not by the polycations. The data suggest that one of the functions (and possibly the only function) of cell-free extracts derived from spleen tissue is the restoration of oxidative phosphorylation in irradiated organisms. Since this effect can be duplicated by any of several commercially prepared polyanions, it seems unlikely that the spleen has in it a special agent responsible for effecting radiation protection. The data obtained with oxidative phosphorylation are in no way contradictory to the cellular replacement hypothesis of increased survival after exposure to radiation; they merely explain a possible mode of action of humoral agents which are known to have a limited effect on survival. Failure of PES to increase survival under the same conditions in which it recouples phosphorylation would seem to indicate that survival and regeneration of the phosphorylation mechanism are independent processes.

LITERATURE CITED

Allfrey, V. G., and A. E. Mirsky, 1958. Some effects of substituting the deoxyribonucleic acid of isolated cell nuclei with other polyelectrolytes. *Proc. Nat. Acad. Sci.*, **44**: 981–991.

Benjamin, T. L., and H. T. Yost, 1960. The mechanism of uncoupling of oxidative phosphorylation in rat spleen and liver mitochondria after whole-body irradiation. *Rad. Res.*, **12**: 613–625.

Cole, L. J., and M. E. Ellis, 1954. Studies on the chemical nature of the radiation protection factor in mouse spleen. I. Enzymatic inactivation by DNAases and trypsin. *Rad. Res.*, **1**: 347–357.

Ellinger, F., 1956. Further studies with cell-free extracts from mouse spleen on X-ray induced mortality. *Proc. Soc. Exp. Biol. Med.*, **92**: 670–673.

Ellinger, F., 1957. Protection of guinea pigs against radiation death by cell-free mouse spleen extracts. *Science*, **126**: 1179–1180.

Glick, D., 1949. Techniques of Histo- and Cytochemistry. Interscience Publishers, Inc., New York.

Hanson, J. B., 1959. The effect of ribonuclease on oxidative phosphorylation by mitochondria. *J. Biol. Chem.*, **234**: 1303–1306.

Koller, P. C., A. J. S. Davies and S. Doak, 1961. Radiation Chimeras. *In:* Advances in Cancer Research, A. Haddow and S. Weinhouse, Eds., Academic Press, New York. Vol. VI. Pp. 181–291.

Lehninger, A. L., 1959. An endogenous uncoupling and swelling agent in liver mitochondria and its enzymatic formation. *J. Biol. Chem.*, **234**: 2459–2464.

Linnane, A. W., and E. B. Titchener, 1960. Studies of the mechanism of oxidative phosphorylation. VI. A factor for coupled oxidation in the electron transport particle. *Biochem. et Biophys. Acta*, **39**: 469–478.

Panjevac, B., G. Ristic and D. Kanazir, 1958. Recovery from radiation injury by rats following administration of nucleic acids. *Second Internat. Conf. Peaceful Uses Atomic Energy*, **23**: 64–70.

Pinchot, G. B., 1959. A polynucleotide coenzyme of oxidative phosphorylation. III. Mechanism of action. *Biochem. Biophys. Res. Communs.*, **1**: 17.

Schneider, W., 1948. Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. Biol. Chem.*, **176**: 259–266.

van Bekkum, D. W., 1957. The effects of X-rays on phosphorylation *in vivo*. *Biochim. et Biophys. Acta*, **25**: 487–492.

WOJTCZAK, L., AND A. B. WOJTCZAK, 1960. Uncoupling of oxidative phosphorylation and inhibition of ATP-P_i exchange by a substance from insect mitochondria. *Biochim. et Biophys. Acta,* **39:** 277–286.

YOST, H. T., R. M. GLICKMAN AND L. H. BECK, 1964. Studies on the effects of irradiation of cellular particulates. IV. The time sequence of phosphorylation changes *in vivo*. *Biol. Bull.*, **127:** 173–185.

YOST, H. T., AND H. H. ROBSON, 1959. Studies on the effects of irradiation of cellular particulates. III. The effect of combined radiation treatments on phosphorylation. *Biol. Bull.*, **116:** 498–506.

THE EFFECTS OF MERCAPTOETHANOL UPON FORM AND MOVEMENT OF AMOEBA PROTEUS[1]

ARTHUR M. ZIMMERMAN[2]

Department of Zoology, University of Toronto, Toronto, Canada

Although sol-gel transformations probably underlie the mechanism of amoeboid movement, there is disagreement as to whether the motive force essential for locomotion is initiated by a contraction of the plasmagel (Mast, 1926; Landau *et al.,* 1954) or through a contraction of non-Newtonian endoplasm (plasmasol) in the anterior portion of the cell (Allen, 1961). Several reports (Zimmerman *et al.,* 1958; Zimmerman, 1962b; Landau, 1959) suggest that the ATP system may, indeed, be directly associated with the sol-gel transformations responsible for amoeboid movement. However, the nature of the protein system through which the cell develops its motive force has not been completely elucidated.

Experiments employing mercaptoethanol, a compound having readily available source of —SH groups, have demonstrated that gelated structures within the cell, such as the mitotic apparatus (Mazia and Zimmerman, 1958), and the cortical plasmagel of cleaving eggs (Zimmerman, 1962a, 1964), are markedly altered by the addition of thiol compounds. Since it is generally believed that gelation reactions in amoebae are responsible for the maintenance of amoeboid form and locomotion, presumably mercaptoethanol, by interfering with the sol-gel reactions, should alter the structural characteristics of the plasmagel and thus have an effect upon pseudopodial form and activity.

It is well established that both temperature and pressure exert marked effects on the gelational state of cytoplasmic structures, and these effects can be evaluated quantitatively. The formation of gelated structures within cells appears to represent an endothermic reaction which is accompanied by a volume increase (Marsland, 1956). Thus, decreasing temperature and increasing pressure tend to weaken the plasmagel structure of amoeba by causing a shift in the sol-gel equilibria toward the sol state. In the present study, the effects of mercaptoethanol on the form of amoebae were determined under systematically varying conditions of pressure.

MATERIALS AND METHODS

Culture methods. Actively growing cultures of *Amoeba proteus* were obtained from Dr. J. A. Dawson. The amoebae were cultured by the method of Brandwein (1935), modified by the elimination of agar. The cultures were grown at 18–20° C. in the dark, at pH 6.9.

---

[1] This work was supported in part by Grant GM 07157-03, 04, 05 from the Division of General Medical Sciences, United States Public Health Service.

[2] Part of this work was conducted while the investigator was affiliated with the Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, N. Y.

Pressure-temperature equipment. The pressure apparatus was patterned after one designed by Marsland (1950) with certain modifications. The microscope-pressure chamber permits cells to be observed at magnifications up to 600 × while being subjected to pressures up to 20,000 lbs./in.^{2}. Pressure is developed by means of an Aminco pressure pump at the rate of 5000 lbs./in.^{2}/ second. The pressure is released virtually instantaneously by means of a needle valve. The microscope-pressure chamber, as well as all glassware and test solutions, was housed in the temperature control chamber. The temperature chamber permits the temperature to be set at any level between $-5°$ and 60° C., with a maximum internal variation of $\pm 0.2°$. In the present experiments the temperature was kept constant at 20° C.

Immersion procedure. For each experiment about 50–100 actively streaming amoebae were washed with Brandwein solution and placed into mercaptoethanol so that the final dilution of mercaptoethanol was 10^{-1}–10^{-6} M. The amoebae were then transferred to a small lucite chamber and placed into the temperature-equilibrated pressure bomb. After a one-hour immersion in mercaptoethanol, the cells were subjected to pressure. During the 20-minute pressure treatment, the cells were kept under observation. The percentage of amoebae with some persisting pseudopodia, as compared with amoebae which were completely spherical, was established.

Chemicals. The 2-mercaptoethanol ($HSCH_2CH_2OH$) was obtained from Eastman Organic Chemicals, Rochester, N. Y. Fresh solutions of mercaptoethanol in Brandwein solution were made up daily and equilibrated at the desired temperature prior to use.

RESULTS

Concentration series. Before investigating the effects of pressure on mercaptoethanol-treated amoebae, it was necessary to find a concentration of mercaptoethanol in which the amoebae would continue to display their normal form and mobility. Amoebae, at 20° C., were immersed into mercaptoethanol at various concentrations ranging from 10^{-1} to 10^{-5} M. At a concentration of 10^{-1} M, the amoebae reacted quickly to the drug. The pseudopodia retracted and protoplasmic streaming decreased. When the amoebae were placed in a concentration of 10^{-2} M about 50% retracted their pseudopodia. However, within a short time most of the amoebae immersed in 10^{-2} M mercaptoethanol developed short pseudopodia and streaming was observed. When the amoebae were immersed in mercaptoethanol solutions with concentrations ranging from 10^{-3} to 10^{-5} M there was no retraction of pseudopodia and amoeboid movement was not modified perceptibly.

After three hours' incubation in 10^{-1} M mercaptoethanol, many of the cells underwent cytolysis while the remaining amoebae retracted their pseudopodia and did not exhibit any protoplasmic activity. Following a three-hour immersion in 10^{-2} M mercaptoethanol, about 50–70% of the amoebae were stellate, with short pseudopodia and with a darkened central mass of cytoplasmic granules. Twenty-four hours later, surviving amoebae in 10^{-1} and 10^{-2} M mercaptoethanol were rounded and did not exhibit protoplasmic streaming, and a large percentage of the cells had undergone cytolysis. However, the amoebae in the more dilute mercaptoethanol concentration (10^{-3} to 10^{-5} M) for a period of 24 hours exhibited apparently normal amoeboid movement and active protoplasmic streaming.

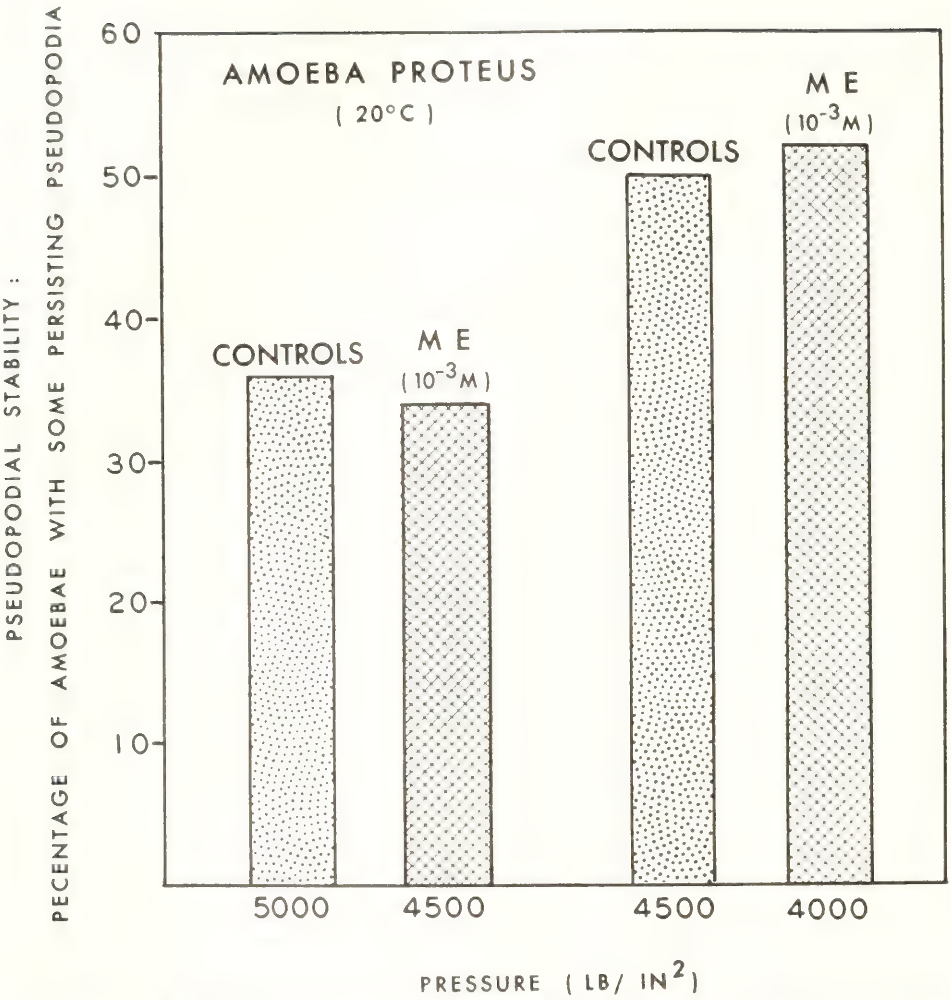


FIGURE 1. The effects of mercaptoethanol (10^{-3} M) on pseudopodial stability in *Amoeba proteus.* In each experiment the values give the percentage of amoebae with definitely persisting pseudopodia after a compression period of 20 minutes at the designated pressure.

Pressure studies. Previously it was reported (Marsland and Brown, 1936; Landau *et al.*, 1954) that the pseudopodia of amoebae become unstable and the amoebae round up into a spherical shape when they are subjected to high hydrostatic pressure. From pressure-centrifuge experiments it was established that the pressure level necessary to induce this rounding is a function of the relative strength of the plasmagel of the amoeba. Consequently, the rounding of the amoeba under pressure was employed as an index of plasmagel strength and the effects of mercaptoethanol were evaluated upon the pseudopodial stability.

Having established that amoebae exhibit apparently normal form and activity for a 24-hour period in 10^{-3} M mercaptoethanol, this concentration was chosen for a series of pressure experiments. In each experiment about 50–100 amoebae were placed in 10^{-3} *M* solution of mercaptoethanol which was previously equilibrated to 20° C. After an incubation of one hour, the amoebae were subjected to a pressure of 4000–5000 lbs./in.^{2} for a period of 20 minutes. Prior to the application of pressure and during the pressure treatment the amoebae were kept under constant observation. Counts were made to determine the percentage of the specimens which lost all pseudopodia and became spherical as compared with

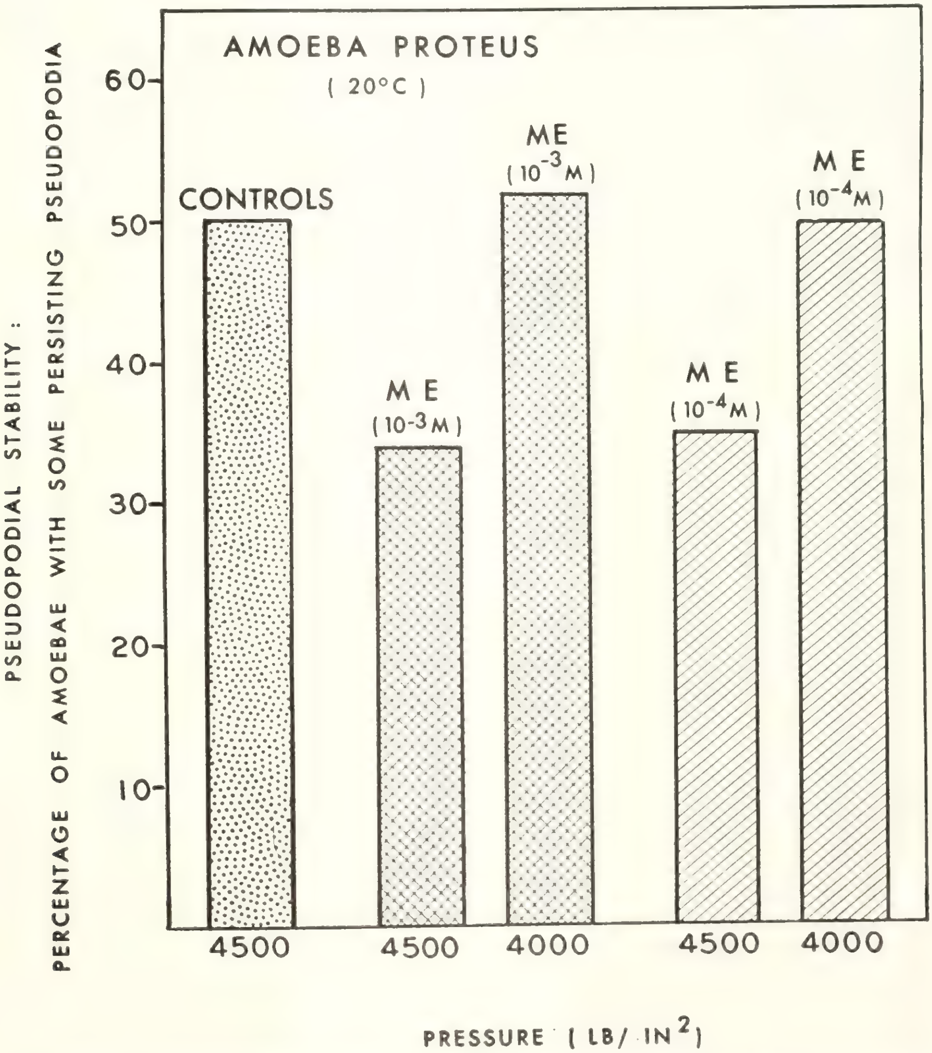


FIGURE 2. The comparative effects of 10^{-3} *M* mercaptoethanol and 10^{-4} *M* mercaptoethanol on pseudopodial stability. In each experiment the values show the percentage of amoebae with definitely persisting pseudopodia after a standard compression period of 20 minutes at 4500 lbs./in.^{2} and 4000 lbs./in.^{2}.

those which retained distinct vestiges of the pseudopodia. Following the pressure treatment the specimens were decompressed. Shortly after decompression, essentially normal form and streaming were evident.

As shown in Figure 1, at a pressure of 4500 lbs./in.^{2}, 50% of the control specimens retained some persisting pseudopodia following 20 minutes of pressure. However, when the amoebae were immersed in 10^{-3} M mercaptoethanol, only 34% of the specimens retained pseudopodia. When the pressure was reduced to 4000 lbs./in.^{2}, the pseudopodial stability was comparable to that of the controls at the higher pressure level. Similar pseudopodial stability characteristics were found at other pressures. At 5000 lbs./in.^{2}, 35% of the control amoebae retained some pseudopodia after 20 minutes of pressure, but in the presence of 10^{-3} M mercaptoethanol, only 27% of the cells retained their pseudopodia. However, when the pressure was lowered to 4500 lbs./in.^{2}, the mercaptoethanol-treated amoebae exhibited stability values equivalent to those of the controls at 5000 lbs./in.^{2}. Consistently the percentage of mercaptoethanol amoebae with persisting pseudopodia was less than that of the control amoebae at each pressure level tested. This difference in stability indicates a pressure differential of 500 lbs./in.^{2}.

TABLE I

Pressure-induced solation of Amoeba proteus *in mercaptoethanol solutions*

Pressure lbs./in.^{2}	Percentage of fully rounded quiescent specimens					
	Controls	Mercaptoethanol concentration				
		$10^{-2}M$	$10^{-3}M$	$10^{-4}M$	$10^{-5}M$	$10^{-6}M$
5000	64 (409)	78 (27)	73 (48)	—	—	—
4500	50 (1827)	68 (258)	66 (301)	65 (107)	66 (134)	45 (150)
4250	38 (100)	51 (72)	61 (358)	—	—	—
4000	28 (71)	59 (64)	48 (79)	50 (54)	—	—

* In each experiment, the percentage of completely rounded quiescent specimens, without any persisting pseudopodia, was determined after a standardized compression period of 20 minutes. The figures in parentheses indicate the total numbers of specimens observed in each case.

Pseudopodial stability in 10^{-3} and 10^{-4} M mercaptoethanol proved to be essentially the same. As is shown in Figure 2, 34–35% of the amoebae retained some pseudopodia at 4500 lbs./in.^{2} in both 10^{-3} and 10^{-4} M mercaptoethanol solutions, whereas 50–52% retained pseudopodia when immersed in 10^{-3} or 10^{-4} M solutions at 4000 lbs./in.^{2}. This latter degree of stability for the mercaptoethanol-treated amoebae at 4000 lbs./in.^{2} was equivalent to that of control amoebae at 4500 lbs./in.^{2} (Table I).

In a further study on the effect of varying concentrations on pseudopodial stability, amoebae in varying concentrations of mercaptoethanol (10^{-2}–10^{-6} M) were exposed to a pressure of 4500 lbs./in.^{2}. As shown in Figure 3, the pseudopodial stability of amoebae in 10^{-2}–10^{-5} M solutions was distinctly less than that of the controls. At a concentration of 10^{-6} M, however, pseudopodial stability was similar to that of the controls. In fact, a small increase in stability was indicated.

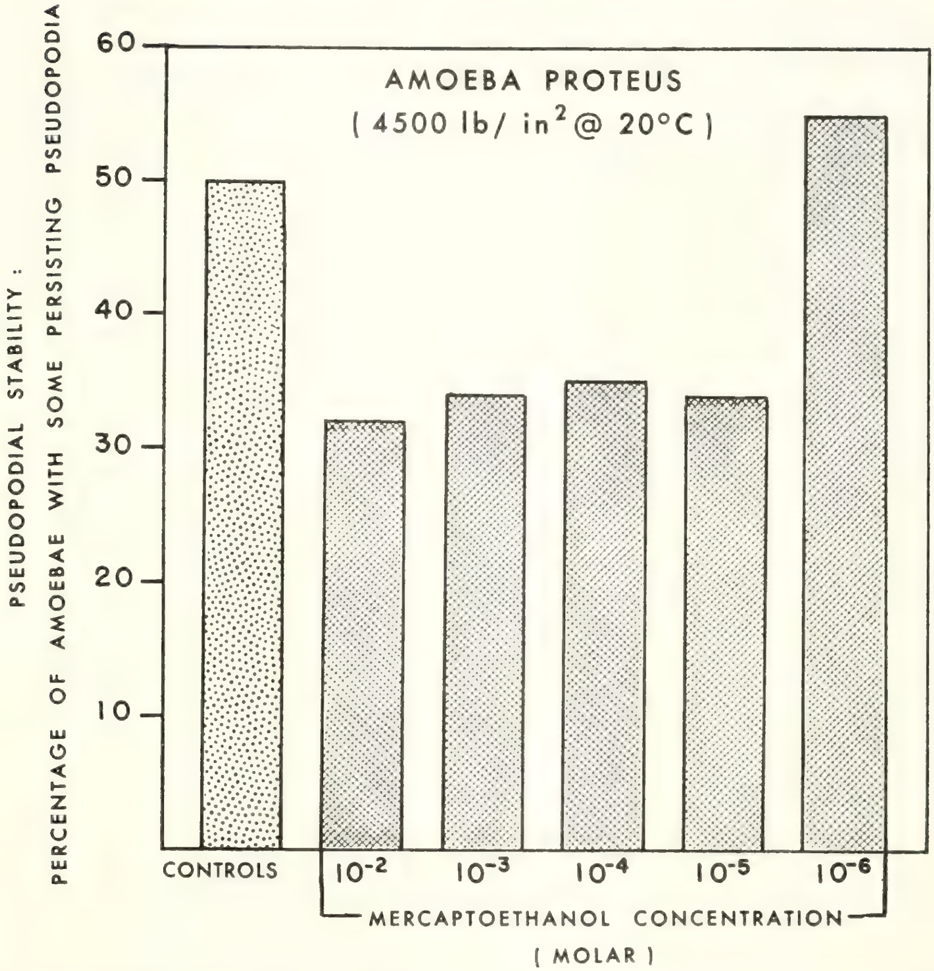


FIGURE 3. The effects of varying concentrations of mercaptoethanol on pseudopodial stability. In each experiment not less than 100–150 specimens were used and each value represents the percentage of non-rounded specimnes with some definitely persisting pseudopodia, after a 20-minute exposure to a pressure of 4500 lbs./in.^{2}, at 20° C.

DISCUSSION

In general, the results support the hypothesis that a proper balance of thiol and disulfide groups may be essential for the formation and maintenance of gelated structures in the cell. In the amoeba, the results support the supposition that high concentrations of $-SH$ groups interfere with the establishment of labile protein linkages, modifying protoplasmic gelation reactions, and ultimately inhibiting amoeboid movement (see, also, Mazia and Zimmerman, 1958).

There is strong evidence that $-SH$ groups of constituent protein molecules may play an important role in the assembly of macromolecular complexes. The structural integrity of many different systems has been shown to be a function of sulfur linkages and the availability of —SH groups. Amberson and colleagues (1957) demonstrated that Δ-myosin may be separated from Δ-protein by the action of salyrgan which, presumably, acts on the thiol groups of the Δ-protein. Further evidence as to the importance of sulfhydryl groups in the maintenance of structural integrity has been reported by Madsen and Cori (1956). These workers showed that p-chloromercuribenzoate is capable of breaking the enzyme α-phosphorylase into smaller fractions, and that this reaction is readily reversed by the addition of cysteine. The importance of the balance of thiol groups in protoplasmic gel structure has also been demonstrated in relation to the formation and the maintenance of the mitotic apparatus (Mazia and Zimmerman, 1958) and in studies on the structural characteristics in cortical plasmagel of cleaving sea urchin eggs (Zimmerman, 1962a, 1964). Contractility of thread models prepared from fertilized sea urchin eggs is also accompanied by —SH changes in the contractile protein (Sakai, 1962).

The decrease of pseudopodial stability in *Amoeba proteus* following treatment with mercaptoethanol may be explained as an interference with SH⇌SS interactions, necessary for the formation of the gel structure. At high concentrations of mercaptoethanol (*e.g.*, 0.1 *M*), the pseudopodia were not maintained. This probably indicates a lowering of plasmagel strength, since pressure studies have shown that below certain values for plasmagel rigidity, the pseudopodia are not maintained. When lower concentrations of mercaptoethanol were used (10^{-2}–10^{-5} *M*) the gel formed was apparently rigid enough to support the pseudopodia and permit pseudopodial formation. However, the pressure studies indicate that the gel structure is not as strong as that of the control cells. Although pseudopodial stability was lower with mercaptoethanol treatment (10^{-2}–10^{-5} *M*), it remained approximately the same within this concentration range. When the concentration was decreased further, however, pseudopodial stability returned to normal.

There is strong evidence to support the concept that contractility in amoeba is dependent upon an ATP system (Zimmerman *et al.*, 1958). Recently, Simard-Duquesne and Couillard (1962a, 1962b) have prepared glycerinated models of amoeba which are activated in the presence of magnesium and ATP. In addition, they have prepared ATP-ase from amoeba and this resembles myosin ATP-ase. Further support for the assumption that the plasmagel layer of the amoeba represents an ATP-sensitive contractile system can be found in the recent work of Sells *et al.* (1961) and Zimmerman (1962b). These investigators demonstrated ATP-ase activity on the surface of the amoeba. Abe (1963) has reported that protoplasmic streaming in amoeba may be reversibly blocked with p-chloromercuribenzoate, which probably interferes with SS⇌SH interactions in the cell. These studies suggest, therefore, that physiological activity in the thiol-sensitive plasmagel structure of *Amoeba proteus* may be dependent upon the energy transactions of the ATP system.

The author wishes to express his thanks to Professor Douglas Marsland for his helpful criticism in the preparation of the manuscript. The author acknowledges with gratitude the technical assistance of Miss Sondra C. Corff and Mrs. Regina Schuel.

SUMMARY

The effects on *Amoeba proteus* of mercaptoethanol solutions of varying concentrations were studied. In 10^{-1} *M* mercaptoethanol the amoebae lose their pseudopodia and after several hours undergo cytolysis. Amoebae immersed in lesser concentrations (10^{-3} to 10^{-5} *M*) maintain an apparently normal form and normal movement. The effects of mercaptoethanol on the stability of the pseudopodia, as indicated by their resistance to the solational action of high pressure, showed a distinct loss of pseudopodial stability. The decreased stability of the pseudopodia induced by these mercaptoethanol solutions indicated a pressure differential of 500 lbs./in.^{2} (at 20° C.), in comparison with the pseudopodial stability of control amoebae. This loss of stability in the mercaptoethanol-treated amoebae is interpreted as a weakening of the plasmagel structure of the amoeba. The experiments also suggest that the sol-gel equilibrium in amoeba is a thiol-sensitive system and that interference with this system inhibits protoplasmic gelation and reduces pseudopodial stability.

LITERATURE CITED

ABE, S., 1963. The effect of *p*-chloromercuribenzoate on amoeboid movement, flagellar movement and gliding movement. *Biol. Bull.*, **124**: 107–114.

ALLEN, R. D., 1961. A new theory of ameboid movement and protoplasmic streaming. *Exp. Cell Res., Suppl.*, **8**: 17–31.

AMBERSON, W. R., J. I. WHITE, H. B. BENSUSAN, S. HIMMELFARB AND B. E. BLANKENHORN, 1957. Δ protein, a new fibrous protein of skeletal muscle: preparation. *Amer. J. Physiol.*, **188**: 205–226.

BRANDWEIN, P. F., 1935. The culturing of fresh water protozoa and other small invertebrates. *Amer. Natural.*, **69**: 628–632.

LANDAU, J. V., 1959. Sol-gel transformations in amoebae. *Ann. N. Y. Acad. Sci.*, **78**: 487–500.

LANDAU, J. V., A. M. ZIMMERMAN AND D. A. MARSLAND, 1954. Temperature-pressure experiments on *Amoeba proteus;* plasmagel structure in relation to form and movement. *J. Cell. Comp. Physiol.*, **44**: 211–232.

MADSEN, N. B., AND C. F. CORI, 1956. The interaction of muscle phosphorylase with *p*-chloromercuribenzoate. *J. Biol. Chem.*, **223**: 1055–1065.

MARSLAND, D. A., 1950. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. Comp. Physiol.*, **36**: 205–227.

MARSLAND, D. A., 1956. Protoplasmic contractility in relation to gel structure. *Int. Rev. Cytol.*, **5**: 199–227.

MARSLAND, D. A., AND D. E. S. BROWN, 1936. Amoeboid movement at high hydrostatic pressure. *J. Cell. Comp. Physiol.*, **8**: 167–178.

MAST, S. O., 1926. Structure, movement, locomotion and stimulation in amoeba. *J. Morph.*, **41**: 347–425.

MAZIA, D., AND A. M. ZIMMERMAN, 1958. SH compounds in mitosis. II. The effect of mercaptoethanol on the structure of the mitotic apparatus in sea urchin eggs. *Exp. Cell Res.*, **15**: 138–153.

SAKAI, H., 1962. Studies on sulfhydryl groups during cell division of sea urchin egg. V. Change in contractility of the thread model in relation to cell division. *J. Gen. Physiol.*, **45**: 427–438.

SELLS, B. H., N. SIX AND J. BRACHET, 1961. The influence of the nucleus upon adenosine triphosphatase activity in *Amoeba proteus*. *Exp. Cell Res.*, **22**: 246–256.

SIMARD-DUQUESNE, N., AND P. COUILLARD, 1962a. Ameboid movement. I. Reactivation of glycerinated models of *Amoeba proteus* with adenosinetriphosphate. *Exp. Cell Res.*, **28**: 85–91.

Simard-Duquesne, N., and P. Couillard, 1962b. Ameboid movement. II. Research of contractile proteins in *Amoeba proteus*. *Exp. Cell Res.*, **28**: 92-98.

Zimmerman, A. M., 1962a. The effects of mercaptoethanol on cleaving eggs of *Arbacia punctulata*. *Biol. Bull.*, **123**: 518–519.

Zimmerman, A. M., 1962b. Action of ATP on amoeba. *J. Cell. Comp. Physiol.*, **60**: 271–280.

Zimmerman, A. M., 1964. Effects of mercaptoethanol on the furrowing capacity of *Arbacia* eggs. *Biol. Bull.*, **127**: 345–352.

Zimmerman, A. M., J. V. Landau and D. Marsland, 1958. The effects of adenosine triphosphate and dinitro-o-cresol upon the form and movement of *Amoeba proteus*. A pressure study. *Exp. Cell Res.*, **15**: 484–495.

INDEX

A

ATPase, extraction of from sea urchin and fish sperm tails, 381 (abstract).

ATPase activities and adenylate kinase in Spisula and Asterias eggs, 374 (abstract).

Abbott, B. C. See M. J. Pak, 382 (abstract).

Abstracts of papers presented at the Marine Biological Laboratory, 353.

Acartia, salinity tolerances of, 108.

Acceleration of recovery of phosphorylation in irradiated cellular particulates, 173.

Acid phosphatase in invertebrate eggs, localization of, 389 (abstract).

Acmaea, development of, 294.

Acoustic orientation of bats, 478.

Activation energy of mollusc ribonucleases, 489.

Activity patterns of Spiochaetopterus, 397.

Adaptation of magnetoreceptive mechanism of snails to geomagnetic strength, 221.

Adelman, W. J., Jr., and F. M. Dyro. Relation of hyperpolarizing response to potassium conductance in internally perfused squid axons, 361 (abstract).

Adkisson, P. L. See C. M. Williams, 511.

Adult male Pyrrhocoris, metabolism of, 499.

Aedes, effects of apholate on, 119.

Aggregation of slime mold, effect of light on, 85.

Alaska, sun compass orientation of pigeons displaced to, 154.

Alkylating agent, effect of on mosquito reproduction, 119.

Allatectomized Pyrrhocoris, metabolism of, 499.

Allen, M. J. Embryological development of the syllid, Autolytus, 187.

Amino acid receptors in crabs, 428.

Amino acids in Nephtys coelomic fluid, 63.

Amoeba, effects of mercaptoethanol on form and movement of, 538.

Amphibian development, anaerobic glycolysis in, 256.

Amphibian epidermis, sequential induction of, 413.

Amphibian larvae, serum antibody synthesis in, 232.

Amphibious crabs, water and salt regulation in, 447.

Anaerobic development in amphibian development, 256.

Anatomy of Aedes sexual apparatus, 324.

Anatomy of Spiochaetopterus, 397.

Anaerobiosis, effect of on snail behavior, 271.

Annelid, biochemistry of coelomic fluid of, 63.

Annelid, development of (Autolytus), 187.

Annelid, tube-building and feeding in, 397.

Annual Report of the Marine Biological Laboratory, 1.

Anomuran, intermolt cycle of, 97.

Anoxia, effect of on snail behavior, 271.

Antennary glands of crabs, role of in salt and water regulation, 447.

Antherea, photoperiodic control of diapause in, 511.

Antibody synthesis in bullfrog larvae, 232.

Antimitotic effects of heavy water, reversal of by high pressure, 356, 380 (abstracts).

Anuran larvae, immunology of, 232.

Apholate, effect of on mosquitoes, 119.

Aquatic crabs, water and salt regulation in, 447.

Arbacia eggs and oocytes, jelly coat, 132.

Arctic Circle, sun compass orientation of pigeons displaced north of, 154.

Auclair, W. On the chromosome number of Arbacia, 359 (abstract).

Austin, C. R. See J. F. Fallon, 369 (abstract).

Autolytus, embryology of, 187.

Autoradiography of Strongylocentrotus gut, 280.

B

Background, role of in pigmentation of Uca, 239.

Bacterial infection of Limulus, 390 (abstract).

Bang, F. B. See H. Rabin, 385 (abstract).

Barnacle, salinity tolerances of, 108.

Barnes, R. D. Tube-building and feeding in the chaetopterid polychaete, Spiochaetopterus, 397.

Barnhill, R. See R. Guttman, 372 (abstract).

Barnwell, F. H. See F. A. Brown, Jr., 206, 221.

Barth, L. G., and L. J. Barth. Sequential induction of the presumptive epidermis of the Rana gastrula, 413.

Basic dyes, effects of on sea urchin egg jelly coat, 132.

Bauer, G. E., G. Lester and A. Lazarow. Studies on the subcellular sites of protein synthesis in goosefish islet tissue, 361 (abstract).

Beatty, R. A. Density gradient media for mammalian spermatozoa, 354 (abstract).

Beck, L. H. See H. T. Yost, Jr., 173, 526.

Behavior, homing, of northern-displaced pigeons, 154.

Behavior of bat in echolocation of flying insects, 478.

Behavior patterns in copulation of Aedes, 324.

Bennett, M. V. L., M. Giménez, Y. Nakajima and G. D. Pappas. Spinal and medullary nuclei controlling electric organ in the eel, Electrophorus, 362 (abstract).

Biggers, J. D. See B. D. Moore, 381 (abstract).

Billiar, R. B., J. C. Brungard and C. A. Villee. Effects of D-malate on production and activity of L-malate dehydrogenases in developing sea urchin eggs, 362 (abstract).

Biochemistry of Nephtys coelomic fluid, 63.

Bioluminescence in Jamaican fireflies, 159.

Boring mollusks, use of polished shell for testing demineralization activity of, 365 (abstract).

Bownds, D., and G. Wald. The reaction of sodium borohydride with rhodopsin and intermediates of its bleaching, 359 (abstract).

Brain, role of in control of diapause of Antherea, 511.

Brandt, P. W. See P. B. Dunham, 368 (abstract).

Branham, J. M. A method of rating sea urchin sperm motility, 363 (abstract).

Branham, J. M. Senescence of Arbacia sperm, 363 (abstract).

Breeding season of Acmaea, 294.

Breeding season of Autolytus, 187.

Brown, F. A., Jr., F. H. Barnwell and H. M. Webb. Adaptation of the magnetoreceptive mechanism of mud-snails to geomagnetic strength, 221.

Brown, F. A., Jr., H. M. Webb and F. H. Barnwell. A compass directional phenomenon in mud-snails and its relation to magnetism, 206.

Brungard, J. C. See R. B. Billiar, 362 (abstract).

Buccinum, temperature coefficient of ribonucleases of, 489.

Buchsbaum, V. Effects of temperature on reaggregation of sponge cells, 364 (abstract).

Buck, J. B. See H. H. Seliger, 159.

Bullfrog larvae, serum antibody synthesis in, 232.

Burns, E. R. See A. M. Mun, 467.

Bush, L. Distribution and behavior of some marine Turbellaria of the Woods Hole region, 364 (abstract).

C

Calcium concentrations in blood of crabs, 447.

Cancer (crab), water and salt regulation in, 447.

Carbohydrate, fluctuations in, in Cape Cod pond, 395 (abstract).

Carbohydrate distribution in Nephtys coelomic fluid, 63.

Carbon dioxide, effect of on cell aggregation of slime mold, 85.

Carbon dioxide, effect of on Taphius distress syndrome, 271.

Carcinus larvae, salinity tolerances of, 108.

Carden, G. A., III, and H. S. Rosenkranz. Preliminary studies on the DNAs isolated from haploid and diploid cells of Arbacia, 365 (abstract).

Carden, G. A., III. See H. S. Rosenkranz, 387 (abstract).

Carriker, M. R., and D. van Zandt. Use of polished mollusk shell for testing demineralization activity of accessory boring organ of muricid boring gastropods, 365 (abstract).

Carter, J. C. H. A preliminary survey of the calanoid copepods of certain embayments and estuaries of Cape Cod, 365 (abstract).

Cartilage in a marine polychaete, 357 (abstract).

Case, J. Properties of the dactyl chemoreceptors of Cancer, 428.

Cecropia silkworm, possible presence of mRNA in, during diapause, 378 (abstract).

Cell aggregation of slime mold, effect of light on, 85.

Cell division in Paramecium, delay of, induced by fluorophenylalanine, 386 (abstract).

Cell interaction in chick splenomegaly, 467.

Cellular particulates, irradiation of, 173, 526.

Cenozoic barnacle, re-evaluation of, 360 (abstract).
Centrifuge-pressure studies on Arbacia eggs, 345.
Centropages, salinity tolerances of, 108.
Chaetopterid, tube-building and feeding in, 397.
Change in color pattern in Uca, 239.
Changes in biochemistry of Nephtys coelomic fluid, 63.
Chasis, J. See W. Troll, 394 (abstract).
Chelipods of Cancer, chemoreception in, 428.
Chemoreceptors of Cancer, dactyl, properties of, 428.
Chemosterilants, cytogenetic effects of on mosquitoes, 119.
Chemotactant of Campanularia, isolation of, 381 (abstract).
Cheney, R. H. See C. C. Speidel, 353 (abstract).
Chick embryo, cell interaction in splenomegaly of, 467.
Chilonycteris, echolocation of flying insects by, 478.
Chloride fluxes in crayfish muscle fibers, 368 (abstract).
Chorioallantoic grafting in chick embryo, 467.
Chromatography of mollusc ribonucleases, 489.
Chromatography of Nephtys coelomic fluid, 63.
Chromatophorotropins, role of in formation of Uca black pigment, 239.
Chromosome number of Arbacia, 359, 370 (abstracts).
Chromosome number of Autolytus, 187.
Chromosomes of Drosophila, specific puff induction in, by tryptophan, 369 (abstract).
Ciliate, effects of actinomycin D on head regeneration in, 393 (abstract).
Claff, C. L., and A. A. Crescenzi. The shark as an experimental organism, 366 (abstract).
Clark, M. E. Biochemical studies on the coelomic fluid of Nephtys, with observations on changes during different physiological states, 63.
Cleavage of Acmaea, 294.
Cleavage of Arbacia eggs, effects of mercaptoethanol on, 345.
Cleavage of Autolytus, 187.
Cobalt-60 irradiation of rats, 173.
Coefficient, temperature, of mollusc ribonucleases, 489.
Coelomic fluid of Nephtys, biochemistry of, 63.
Collagens, soluble, of dogfish skin, 369 (abstract).
Color change in Uca, 239.
Compass directional phenomenon in mud-snails, 206.
Connell, G. M., and G. Kaley. Evidence for the presence of "renin" in kidneys of marine fish and amphibia, 366 (abstract).
Control, photoperiodic, of diapause in Atherea, 511.
Cooper, E. L., W. Pinkerton and W. H. Hildemann. Serum antibody synthesis in larvae of the bullfrog, Rana, 232.
Cooperstein, S. J. See D. Watkins, 395 (abstract).
Copeland, E. Gas secretion in teleost swimbladder, Fundulus and Opsanus, 367 (abstract).
Copeland, E. Salt-absorbing cells in gills of crabs, Callinectes and Carcinus, 367 (abstract).
Copepods, calanoid, of Cape Cod, 365 (abstract).
Copepods, salinity tolerance of, 108.
Copulation in Aedes, 324.
Cortical changes in cleaving Arbacia eggs, effects of mercaptoethanol on, 345.
Crab, fiddler, color change in, 239.
Crab, intermolt cycle of, 97.
Crab dactyl chemoreceptors, properties of, 428.
Crabs, water and salt regulation in, 447.
Crescenzi, A. See C. L. Claff, 366 (abstract).
Crustacean, intermolt cycle of, 97.
Crustacean, properties of dactyl chemoreceptors of, 428.
Crustacean, water and salt regulation in, 447.
Crustaceans, salinity tolerances of, 108.
Cycle, intermolt, of Petrolisthes, 97.
Cycles of response to photoperiod in dragonfly, 304.
Cyclic aspects of snail orientation, 206.
Cytogenetic effects of chemosterilants in mosquitoes, 119.
Cytokinesis, new concept of mechanism of, 389 (abstract).
Cytokinesis of Arbacia eggs, effects of mercaptoethanol on, 345.

D

DNA in Arbacia, 394 (abstract).
DNA base composition of a red alga, Griffithsia, 375 (abstract).
DNA of dogfish cornea, 378 (abstract).
DNA molecules, alignment of during Loligo spermiogenesis, 357 (abstract).
DNAs from haploid and diploid Arbacia cells, 365 (abstract).
DNase of Griffithsia, separation of from phycoerythrin, 383 (abstract).
Dactyl chemoreceptors of Cancer, properties of, 428.

Darkness, effect of on cell aggregation of slime mold, 85.
Darkness, role of in control of diapause of Antherea, 511.
Darkness, effect of on dragonfly nymphs, 304.
Darkness, effect of on orientation of northern-displaced pigeons, 154.
Day-length, effect of on dragonfly metamorphosis, 304.
Day-length, role of in diapause of silkworms, 511.
Decapod crustacean, intermolt cycle of, 97.
Density gradient media for mammalian sperm, 354 (abstract).
Developing amphibian, immunology of, 232.
Developing Arbacia, amino acid incorporation by isolated mitochondrial fractions of, 386 (abstract).
Developing chick, cell interaction in splenomegaly of, 467.
Development of Acmaea, 294.
Development of amphibian, anaerobic glycolysis in, 256.
Development of Antherea, 511.
Development of Autolytus, 187.
Development of dragonfly, 304.
Development of heat-treated Arbacia eggs, 370 (abstract).
Development of Pinnotheres, 384 (abstract).
Development of Rana epidermis in vitro, 413.
Diapause, insect, physiology of, 511.
Diapause of dragonfly, effect of photoperiod on, 304.
Diapause of Pyrrhocoris, metabolism during, 499.
Digestive gland ribonucleases of molluscs, 489.
Diphotus, flash patterns in, 159.
Directional phenomenon in snails, relation of to magnetism, 206, 221.
Disulfide groups, importance of in cleavage of Arbacia eggs, 345.
Disulfide groups, importance of in form and mobility of amoebae, 538.
Division of Arbacia eggs, effects of mercaptoethanol on, 345.
Dixit, P. K. See D. Watkins, 395 (abstract).
Donor-host cell interaction in chick splenomegaly, 467.
"Dopa" as possible melanin precursor in Uca, 239.
Dragonfly, life-history and photoperiodic responses of, 304.
Driscoll, A. L. Relationship of mesh opening to faunal counts in a quantitative benthic study of Hadley Harbor, 368 (abstract).
Driscoll, A. L. See R. H. Parker, 360 (abstract).
Drosophila, echolocation of by bats, 478.
Dugesia homogenates, succinoxidase activity in, 317.
Dunham, P. B., J. P. Reuben and P. W. Brandt. Chloride fluxes in crayfish muscle fibers after vesiculation of transverse tubular system and after treatment with procaine, 368 (abstract).
Dyes, effects of on sea urchin egg jelly coat, 132.
Dyro, F. M. See W. J. Adelman, 361 (abstract).

E

Ecdysis cycle of Petrolisthes, 97.
Echinoderm eggs, effects of mercaptoethanol on furrowing capacity of, 345.
Echinoderm eggs and oocytes, jelly coat of, 132.
Echinoderm gut, histochemistry of, 280.
Echolocation of flying insects by Chilonycteris, 478.
Ecology of Nephtys, in relation to biochemistry of coelomic fluid, 63.
Effects of chemosterilants on mosquitoes, 119.
Effects of gases on distress syndrome in Taphius, 271.
Effects of mercaptoethanol on form and movement in Amoeba, 538.
Effects of mercaptoethanol on furrowing capacity of marine eggs, 345.
Egg production in apholate-treated mosquitoes, 119.
Eggs of Acmaea, development of, 294.
Eggs, Arbacia, effects of mercaptoethanol on furrowing capacity of, 345.
Eggs of sea urchin, jelly coat of, 132.
Electron microscopy of Nereis gamete activation reactions, 369 (abstract).
Electrophysiology of Cancer dactyl chemoreceptors, 428.
Electrophysiology of eel spinal and medullary nuclei, 362 (abstract).
Elminius, salinity tolerances of, 108.
Embryo chick, cell interaction in splenomegaly of, 467.
Embryological development of Autolytus, 187.
Embryology of Acmaea, 294.
Embryology of Rana epidermis in vitro, 413.
Endocrine mechanism for photoperiodic control of diapause in Antherea, 511.
Endocrinology of Pyrrhocoris, 499.
Energetics of hybrid frog development, 256.
Environmental factors, role of in cell aggregation of slime mold, 85.

Environmental and faunal variability, small-scale, preliminary quantitative study of, 360 (abstract).
Enzyme activity in planarian homogenates, 317.
Enzyme kinetics of gastropod ribonucleases, 489.
Epidermis of Rana gastrula, sequential induction of, 413.
Estuarine planktonic crustaceans, salinity tolerances of, 108.
Eyestalkless Uca, studies on pigment of, 239.

F

Fallon, J. C., and C. R. Austin. A fine-structure study of the activation reactions of Nereis gametes, 369 (abstract).
Farmanfarmaian, A. See C. Rundles, 387 (abstract).
Fasciolaria, temperature coefficients of ribonucleases of, 489.
Fastie, W. G. See H. H. Seliger, 159.
Feather inhibition in fluorouracil-treated chick embryos, 467.
Fecundity of mosquitoes, effects of apholate on, 119.
Fedoroff, N., and R. Milkman. Specific puff induction by tryptophan in Drosophila salivary chromosomes, 369 (abstract).
Feeding in Spiochaetopterus, 397.
Fertility of mosquitoes, effect of apholate on, 119.
Fertilization in Acmaea, 294.
Fertilization in Autolytus, 187.
Fibrin polymerization, inhibitors of, 379 (abstract).
Fiddler crab, morphological color change in, 239.
Fireflies, Jamaican, flash patterns in, 159.
Fishman, L., and M. Levy. A comparison of soluble dogfish skin collagens, 369 (abstract).
Fishman, L. See W. Troll, 394 (abstract).
Flash patterns in Jamaican fireflies, 159.
Fluorouracil (5-), role of in chick splenomegaly, 467.
Flying insects, echolocation of by bat, 478.
Form of Amoeba, effect of mercaptoethanol on, 538.
Frog embryo homogenates, anaerobic glycolysis in, 256.
Frog larvae, serum antibody synthesis in, 232.
Fruit flies, echolocation of by bats, 478.
Furrowing capacity of marine eggs, effects of mercaptoethanol on, 345.

G

Gaffkya, infection of Homarus with, 385 (abstract).
Gametes of Acmaea, methods for obtaining, 294.
Gametes of Autolytus, obtaining of, 187.
Gamma irradiation of rats, 173.
Gases, effect of on Taphius behavior, 271.
Gastropod behavior, effect of gases on, 271.
Gastropod ribonucleases, temperature coefficients of, 489.
Gastrula of Rana, sequential induction in, 413.
Gebelein, C. D. See W. R. Taylor, 393 (abstract).
Gecarcinus, water and salt regulation in, 447.
Geilenkirchen, W. L. M. The cleavage schedule and the development of Arbacia eggs as separately influenced by heat shock, 370 (abstract).
Gelation reaction in Amoeba, effects of mercaptoethanol on, 538.
Gelation reaction in Arbacia eggs, effects of mercaptoethanol on, 345.
Genital apparatus of Aedes, 324.
Geomagnetism, effect of on orientation of snails, 206, 221.
German, J. The chromosomal complement of blastomeres in Arbacia, 370 (abstract).
Giménez, M. See M. V. L. Bennett, 362 (abstract).
Gland cells in sea urchin gut, 280.
Glickman, R. M. See H. T. Yost, Jr., 173.
Glycolysis, anaerobic, in amphibian development, 256.
Golgi bodies, possible role of in secretion processes of sea urchin gut, 280.
Golgi saccules in association with endoplasmic reticulum of rat spinal ganglia, 358 (abstract).
Gorovsky, M. A. Heat denaturation studies of Arbacia nucleoproteins, 371 (abstract).
Gradients, photoperiodic, role of in control of silkworm diapause, 511.
Grapsus, water and salt regulation in, 447.
Green, J. P. Morphological color change in the fiddler crab, Uca, 239.
Gregg, J. R., J. J. MacIsaac and M. A. Parker. Anaerobic glycolysis in amphibian development. Homogenates, 256.
Gross, W. J. Trends in water and salt regulation among aquatic and amphibious crabs, 447.
Growth of Pyrrhocoris, metabolism during, 499.
Growth-inhibiting factors from Mercenaria, characteristics of, 388 (abstract).
Grundfest, H. See Y. Nakamura, 382 (abstract).

Gut mucopolysaccharides of Strongylocentrotus, 280.

Guttman, R., and R. Barnhill. Temperature characteristics of excitation in squid axon, 372 (abstract).

H

Habitat of crabs, in relation to water and salt regulation, 447.

Hallberg, R. L. A qualitative study of the hatching enzyme in the sea urchin, Arbacia, 372 (abstract).

Hand, G., Jr., and R. Maggio. RNA metabolism during maturation and early development in Asterias, 372 (abstract).

Harry, H. W., and J. B. Senturia. The effect of nitrogen, oxygen and carbon dioxide in producing the distress syndrome in Taphius, 271.

Hauschka, S. D. Hyaline membrane lysis in Mytilus eggs, 373 (abstract).

Heart of shark as experimental material, 366 (abstract).

Heat, effect of on cell aggregation of slime mold, 85.

Heat denaturation studies on Arbacia nucleoproteins, 371 (abstract).

Hegab, E. S. See J. A. Miller, 380 (abstract).

Hemigrapsus, water and salt regulation in, 447.

Higashino, S. Analysis of the biological excitable membrane by means of voltage-current-time characteristics, 355 (abstract).

Hildemann, W. H. See E. L. Cooper, 232.

Hiramoto, Y. Further studies on the cell division without mitotic apparatus in sea urchin eggs, 357 (abstract).

Hiramoto, Y. Mechanical properties of the starfish oocyte during maturation divisions, 373 (abstract).

Hiramoto, Y. See D. Marsland, 380 (abstract).

Histochemistry of Strongylocentrotus gut, 280.

Histology of exoskeleton formation in Petrolisthes, 97.

Histology of gamma-irradiated rat tissues, 173.

Holland, N. D., and Sr. A. Nimitz. An autoradiographic and histochemical investigation of the gut mucopolysaccharides of the purple sea urchin, Strongylocentrotus, 280.

Homing pigeons, sun compass orientation of, when displaced north of Arctic Circle, 154.

Homogenates of amphibian embryos, anaerobic glycolysis in, 256.

Hormonal action in Pyrrhocoris, 499.

Hormone control of diapause in Antherea, 511.

Horwitz, B. A., and L. Nelson. Adenylate kinase and ATPase activities in Spisula and Asterias eggs, 374 (abstract).

Horwitz, B. A., and L. Nelson. Rates of oxygen consumption of Arbacia, Asterias and Spisula eggs, 374 (abstract).

Host-donor cell interactions in chick splenomegaly, 467.

Howell, S. H., and M. Nasatir. Preliminary determinations of the DNA base composition by specific densities in the haploid and diploid generations of a red alga, Griffithsia, 375 (abstract).

Humphreys, T., and M. Uehara. The effect of inhibition of energy metabolism and protein synthesis on sponge cell aggregation, 375 (abstract).

Hyaline membrane lysis in Mytilus eggs, 373 (abstract).

Hybrid frog embryo homogenates, anaerobic glycolysis in, 256.

Hydrostatic pressure, effects of on cleavage of Arbacia eggs, 345.

Hydrozoa of Cape Cod, 384 (abstract).

Hyperpolarizing response, relation of to potassium conductance in internally perfused squid axon, 361 (abstract).

I

Immunology of bullfrog larvae, 232.

Induction of presumptive epidermis in Rana gastrula, 413.

Inflammatory changes in microcirculation produced by cationic proteins extracted from lysosomes, 356 (abstract).

Influence of light on cell aggregation of slime mold, 85.

Inhibitors, bacterial and viral, effect of on development and metabolism of Arbacia, 394 (abstract).

Inoué, S. See H. Sato, 357 (abstract).

Insect, effect of apholate on, 119.

Insect, metabolism of, during growth, reproduction and diapause, 499.

Insect diapause, physiology of, 511.

Insects, echolocation of by bat, 478.

Insulin release from toadfish islet tissue, glucose stimulation of, 395 (abstract).

Irradiated Arbacia larvae, motility of, 353 (abstract).

Interaction of cells in chick splenomegaly, 467.

Intermolt cycle of Petrolisthes, 97.

Irradiation of cellular particulates, 173, 526.

J

Jacobsen, A. See L. Lorand, 379 (abstract).

Jaffe, S. See W. Troll, 394 (abstract).

Jamaican fireflies, flash patterns in, 159.

Janoff, A., and C. R. Jones. Identification of lysosomes in the brains of lower vertebrates, 376 (abstract).

Janoff, A., and B. W. Zweifach. Production of inflammatory changes in the microcirculation by cationic proteins extracted from lysosomes, 356 (abstract).

Jelly coat of sea urchin eggs and oocytes, properties of, 132.

Jenkins, M. M. See W. L. Mengebier, 317.

Jones, C. R. See A. Janoff, 376 (abstract).

K

Kahn, A. J. The influence of light on cell aggregation in Polysphondylium, 85.

Kaley, G. See G. M. Connell, 366 (abstract).

Kane, R. E. Structural changes in the mitotic apparatus after isolation, 376 (abstract).

Karasaki, S. The sites of nuclear RNA synthesis during amphibian embryogenesis, 354 (abstract).

Kempton, R. T. Some anatomical features of the elasmobranch kidney, 377 (abstract).

Kempton, R. T. The secretion of phenol red by the smooth dogfish, Mustelus, 377 (abstract).

Kessel, M. M. Reproduction and larval development of Acmaea, 294.

Kidney of elasmobranchs, anatomy of, 377 (abstract).

Kinetics, enzyme, of gastropod ribonucleases, 489.

Krishnakumaran, A., and H. A. Schneiderman. Is there long-lived mRNA in diapausing larvae of the Cecropia silkworm?, 378 (abstract).

Kurup, N. G. The intermolt cycle of an anomuran, Petrolisthes, 97.

L

Lactate production in amphibian embryo homogenates, 256.

Lampyrid fireflies, Jamaican, flash patterns in, 159.

Lance, J. Salinity tolerances of estuarine planktonic crustaceans, 108.

Larvae, mosquito, effects of apholate on, 119.

Larvae of Autolytus, 187.

Larvae of bullfrog, serum antibody synthesis in, 232.

Larvae of copepods, salinity tolerances of, 108.

Larval development of Acmaea, 294.

Larval silkworms, photoperiodic control of diapause in, 511.

Lazarow, A. See G. E. Bauer, 361; D. Watkins, 395 (abstracts).

Lecontea, flash patterns in, 159.

Lens of dogfish, cold-precipitable protein in, 358 (abstract).

Leonards, J. See D. Watkins, 395 (abstract).

Lerman, S., G. Munro and S. Zigman. Preliminary studies on dogfish corneal DNA, 378 (abstract).

Lerman, S. See S. Zigman, 358 (abstract).

Lester, G. See G. E. Bauer, 361 (abstract).

Levine, L., and H. V. Vunakis. Mustelus pepsinogen and its reaction with rabbit anti-pepsinogen, 379 (abstract).

Levy, M. See L. Fishman, 369 (abstract).

Life-history of dragonfly, 304.

Light, effect of on cell aggregation of slime mold, 85.

Light, effect of on dragonfly nymphs, 304.

Light, effect of on orientation of northern-displaced pigeons, 154.

Light, role of in control of diapause in Antherea, 511.

Light production by Jamaican fireflies, 159.

Limpet, development of, 294.

Liver, rat, effects of radiation on, 526.

Liver mitochondria, rat, effect of gamma radiation on, 173.

Lorand, L., A. Jacobsen and R. Schuel. Inhibitors of fibrin polymerization, 379 (abstract).

Lukas, K. See R. H. Parker, 360 (abstract).

Lutz, P. E., and C. E. Jenner. Life-history and photoperiodic responses of nymphs of Tetragoneuria, 304.

Lysosomes in brains of lower vertebrates, 376 (abstract).

M

MacIsaac, J. J. See J. R. Gregg, 256.

Maggio, R. See G. Hand, Jr., 372 (abstract).

Magnesium regulation in crabs, 447.

Magnetism, relation of compass directional effect in snails to, 206, 221.

Magnetoreceptive mechanism of snails, adaptation of to geomagnetic strength, 221.

Malate, D-, effects of on production of L-malate dehydrogenases in developing sea urchin, 362 (abstract).

Males of Pyrrhocoris, metabolism of, 499.

Marine eggs, effects of mercaptoethanol on furrowing capacity of, 345.

Marsland, D. High pressure reversal of the antimitotic effects of heavy water, 356 (abstract).
Marsland, D., and Y. Hiramoto. Partial reversal by high pressure of the antimitotic effects of heavy water on the oocytes of the starfish, Asterias, 380 (abstract).
Mating behavior in Aedes, 324.
McElroy, W. D. See H. H. Seliger, 159.
Mechanical properties of starfish oocyte during maturation divisions, 373 (abstract).
Mechanics of copulation in Aedes, 324.
Mechanism of apholate-induced effects on mosquitoes, 119.
Melanin synthesis in Uca, 239.
Membrane excitation, 355 (abstract).
Mengebier, W. L., and M. M. Jenkins. Succinoxidase activity in homogenates of Dugesia, 317.
Mercaptoethanol, effects of on form and movement of Amoeba, 538.
Mercaptoethanol, effects of on furrowing capacity of marine eggs, 345.
Metabolism of irradiated rat cellular particulates, 173.
Metabolism of planarian homogenates, 317.
Metabolism of Pyrrhocoris, 499.
Metamorphosis of dragonfly, in relation to day-length, 304.
Milkman, R. See N. Fedoroff, 369 (abstract).
Miller, J. A., E. S. Hegab and F. S. Miller. Succinic dehydrogenase activity in tubularian development, 380 (abstract).
Miller, R. L. Isolation of the chemotactant of Campanularia, 381 (abstract).
Mitochondria, rat, effect of gamma irradiation of, 173, 526.
Mitosis in apholate-treated mosquitoes, 119.
Mitosis of Arbacia eggs, effects of mercaptoethanol on, 345.
Mitosis in sea urchin eggs without mitotic apparatus, 357 (abstract).
Mitotic apparatus, structural changes in, after isolation, 376 (abstract).
Motility of amoebae, effects of mercaptoethanol on, 538.
Mohri, H. Extraction of ATPase from sea urchin and fish sperm tails, 381 (abstract).
Mollusc, development of, 294.
Mollusc behavior, effect of gases on, 271.
Mollusc ribonucleases, temperature coefficients of, 489.
Molting of crustaceans, effect of salinity on, 108.
Moore, B. D., and J. D. Biggers. In vitro studies on the shedding activity found in Asterias nerve extracts, 381 (abstract).
Morphogenesis and growth of Ulva, effects of salinity, temperature and photoperiodism on, 386 (abstract).
Morphological color changes in fiddler crab, 239.
Morphology of Aedes sexual apparatus, 324.
Morphology of intermolt in Petrolisthes, 97.
Mosquitoes, copulation in, 324.
Mosquitoes, cytogenetic effects of chemosterilants on, 119.
Movement of Amoeba, effects of mercaptoethanol on, 538.
Mucopolysaccharides of Strongylocentrotus gut, 280.
Mud-snails, compass directional phenomena in, 206, 221.
Mun, A. M., and E. R. Burns. Donor-host cell interaction in homologous splenomegaly in the chick, 467.
Munro, G. See S. Lerman, 378 (abstract).
Munro, J. See S. Zigman, 358 (abstract).
Muscle, adductor, of Pecten, band pattern changes in, 391 (abstract).
Muscle, frog sartorius, effect of localized ultrasound on, 396 (abstract).
Muscle, invertebrate, ultraviolet microbeam studies of contraction in, 391 (abstract).
Muscle, voluntary, electrically excitable systems of, 389 (abstract).
Muscle of Limulus heart, direct continuity of sarcolemma with Z-band of, 385 (abstract).
Myofibrils of chick, fluorescent antimyosin labeling in, 390 (abstract).
Myxamoeba, effect of light on cell aggregation of, 85.

N

Na-activation in squid axon and eel electroplaque, selective block of, by tetrodotoxin, 382 (abstract).
Nagle, J. S. Differential sorting of shells in the swash zone, 353 (abstract).
Nagle, J. S. See R. H. Parker, 360 (abstract).
Nakajima, S. See Y. Nakamura, 382 (abstract).
Nakajima, Y. See M. V. Bennett, 362 (abstract).
Nakamura, Y., S. Nakajima and H. Grundfest. Selective block of Na-activation in voltage-clamped squid giant axon and eel electroplaque by tetrodotoxin, 382 (abstract).
Nasatir, M. See S. H. Howell, 375; J. Pawalek, 383 (abstracts).
Nassarius, compass directional phenomena in, 206, 221.

Navigation of pigeons displaced north of Arctic Circle, 154.
Nelson, L. See B. A. Horwitz, 374 (abstracts).
Nephtys, biochemistry of coelomic fluid of, 63.
Nerve cells, induction of in Rana gastrulae, in vitro, 413.
Neural inductions in Rana gastrulae, 413.
Neurophysiology of Cancer dactyl chemoreceptors, 428.
Nimitz, Sr. A. See N. D. Holland, 280.
Nitrogen, effect of on Taphius distress syndrome, 271.
Nitrogen distribution in Nephtys coelomic fluid, 63.
Northern-displaced pigeons, sun compass orientation of, 154.
Novick, A., and J. R. Vaisnys. Echolocations of flying insects by the bat, Chilonycteris, 478.
Novikoff, A. B. GERL, its form and function in neurons of rat spinal ganglia, 358 (abstract).
Nuclei of rat spleen, effects of radiation on, 526.
Nucleic acids isolated from Echinarachnius, 387 (abstract).
Nymphs, dragonfly, life-history and photoperiodic responses of, 304.

O

Oak silkworm, photoperiodic control of diapause in, 511.
Ocypode, water and salt regulation in, 447.
Oocytes of sea urchin, jelly coat of, 132.
Oogenesis of mosquitoes, effect of apholate on, 119.
Orientation, acoustic, of bats, 478.
Orientation phenomena in Nassarius, 206, 221.
Orientation of pigeons displaced north of Arctic Circle, 154.
Osmotic regulation in crabs, 447.
Osmotic relations of estuarine planktonic crustaceans, 108.
Ova, Acmaea, development of, 294.
Ova, Arbacia, effects of mercaptoethanol on furrowing capacity of, 345.
Ovaries of apholate-treated mosquitoes, 119.
Oxidative phosphorylation in irradiated rat cellular particulates, 173.
Oxygen, effect of on Taphius distress syndrome, 271.
Oxygen consumption of Arbacia, Asterias and Spisula eggs, 374 (abstract).
Oxygen consumption of planarian homogenates, 317.
Oxygen consumption of Pyrrhocoris adult males, 499.
Oxygen uptake of irradiated rat cellular particulates, 173, 526.

P

Pachygrapsus, water and salt regulation in, 447.
Pak, M. J., and B. C. Abbott. Mechanics of toadfish swimbladder muscle, 382 (abstract).
Pappas, G. D. See M. V. L. Bennett, 362 (abstract).
Paracentrotus oocytes and eggs, jelly coat of, 132.
Parker, M. A. See J. R. Gregg, 256.
Parker, R. H., J. S. Nagle, A. L. Driscoll and K. Lukas. Preliminary quantitative study of small-scale environmental and faunal variability in Hadley Harbor, 360 (abstract).
Parpart, A. K. The permeability of the plasma membrane of the egg of Arbacia does not alter on fertilization, 383 (abstract).
Patterns of flashing in Jamaican fireflies, 159.
Pawalek, J., and M. Nasatir. Separation of Griffithsia DNase from phycoerythrin, 383 (abstract).
Pearce, J. B. On reproduction in Pinnotheres, 384 (abstract).
Pepsinogen of Mustelus, reaction of with rabbit anti-pepsinogen, 379 (abstract).
Pernyi silkworm, photoperiodic control of diapause in, 511.
Person, P. Cartilage in a marine polychaete, Eudistylia, 357 (abstract).
Petersen, K. W. Some preliminary results of a taxonomic study of the Hydrozoa of the Cape Cod area, 384 (abstract).
Petrolisthes, intermolt cycle of, 97.
Phenylthiourea, effect of on pigment of Uca, 239.
Philpott, D. E. Direct continuity of sarcolemma with Z-band of Limulus heart muscle, 385 (abstract).
Phosphate transfer-storage system, role of in development of amphibian hybrid embryos, 256.
Phosphorylation, recovery of, in irradiated cellular particulates, 526.
Phosphorylation changes in irradiated cellular particulates, 173.
Photinus, flash patterns in, 159.
Photocytes of Jamaican fireflies, 159.
Photoperiodic control of diapause in Antherea, 511.

Photoperiodic responses of dragonfly, 304.
Photuris, flash patterns in, 159.
Physiology of insect diapause. XIV., 511.
Pigeons, orientation of, upon displacement north of Arctic Circle, 154.
Pigment changes in Uca, 239.
Pigments, plant, chromatographic analyses of, 393 (abstract).
Pinkerton, W. See E. L. Cooper, 232.
Planarian homogenates, succinoxidase activity in, 317.
Planktonic crustaceans, salinity tolerances of, 108.
Plasma membrane of Arbacia egg, permeability of, after fertilization, 383 (abstract).
Plasmagel structure in mercaptoethanol-treated amoebae, 538.
Platyhelminth homogenates, succinoxidase activity in, 317.
Polyanions, effects of on recovery of irradiated rat cellular particulates, 526.
Polychaete, biochemistry of, coelomic fluid of, 63.
Polychaete, development of (Autolytus), 187.
Polychaete, tube-building and feeding in, 397.
Polysphondylium, effect of light on cell aggregation in, 85.
Porcelain crab, intermolt cycle in, 97.
Porcellana, salinity tolerances of, 108.
Potassium concentrations in blood of crabs, 447.
Potassium hydroxide, effect of on cell aggregation of slime mold, 85.
Precipitin production in bullfrog larvae, 232.
Pressure, effect of on mercaptoethanol-treated amoebae, 538.
Pressure, effects of on cleavage of Arbacia eggs, 345.
Presumptive epidermis of Rana gastrula, sequential induction of, 413.
Properties of crab dactyl chemoreceptors, 428.
Properties of sea urchin egg jelly coat, 132.
Prosobranch, development of, 294.
Protamin, effect of on sea urchin egg jelly coat, 132.
Protective effects of shielding in irradiation of rats, 173.
Protein synthesis, sites of in goosefish islet tissue, 361 (abstract).
Protozoan, effects of mercaptoethanol on, 538.
Psammechinus oocytes and eggs, jelly coats of, 132.
Pupal diapause in Antherea, photoperiodic control of, 511.
Purple sea urchin, histochemistry of gut of, 280.
Pyrophorus, flash patterns in, 159.
Pyrrhocoris, metabolism of, 499.

Q

Quantitative benthic study of Hadley Harbor fauna, 368 (abstract).

R

RNA metabolism of Asterias eggs, 372 (abstract).
RNA synthesis, sites of in amphibian embryogenesis, 354 (abstract).
Rabin, H., and F. B. Bang. Studies on the infection of the lobster, Homarus, with Gaffkya, 385 (abstract).
Radiation of cellular particulates of rats, 173, 536.
Radiation-sensitivity, nuclear-cytoplasmic relations in, 388 (abstract).
Radiocarbon-labelled tyrosine, studies on, in Uca, 239.
Radiocobalt as source for irradiating cellular particulates, 526.
Radiosulfur, use of in autoradiography of sea urchin gut, 280.
Rai, K. S. Cytogenetic effects of chemosterilants in mosquitoes. II., 119.
Rana embryo homogenates, anaerobic glycolysis in, 256.
Rana gastrula epidermis, sequential induction of, 413.
Rana larvae, serum antibody synthesis in, 232.
Rasmussen, L. Delay of cell division in Paramecium induced by fluorophenylalanine, 386 (abstract).
Rats, irradiation of cellular particulates of, 173, 526.
Rea, I. K. Some effects of salinity, temperature and photoperiodism on the growth and morphogenesis of Ulva, 386 (abstract).
Read, K. R. H. The temperature-coefficients of ribonucleases from two species of gastropod molluscs from different thermal environments, 489.
Recovery of phosphorylation in irradiated cellular particulates, 173, 526.
Regulation of salt and water in crabs, 447.
Relation of compass directional effect in snails to magnetism, 206, 221.
"Renin" in kidneys of marine fish and Amphibia, 366 (abstract).
Reporter, M. C. Amino acid incorporation by isolated mitochondrial fractions of unfertilized Arbacia eggs and late gastrula embryos, 386 (abstract).
Reproduction and larval development of Acmaea, 294.
Reproduction of mosquitoes, effect of apholate on, 119.

Reproduction of Pyrrhocoris, metabolism during, 499.
Respiration of irradiated rat cellular particulates, 173, 526.
Respiration of planarian homogenates, 317.
Respiratory metabolism of Pyrrhocoris, 499.
Reuben, J. P. See P. B. Dunham, 368 (abstract).
Rhodopsin, reaction of sodium borohydride with, and intermediates of its bleaching, 359 (abstract).
Rhythmic aspects of snail orientation, 206.
Ribonucleases, gastropod, temperature coefficients for, 489.
Ribosomal proteins of Arbacia eggs, 392 (abstract).
Richmond, S. S. See H. T. Yost, Jr., 526.
Rosenkranz, H. S., and G. A. Carden III. Unusual deoxyribonucleic acids isolated from the sand dollar, Echinarachnius, 387 (abstract).
Rosenkranz, H. S. See G. A. Carden III, 365; D. Van Praag, 394 (abstracts).
Rundles, C., and A. Farmanfarmaian. Absorption and transport of D-glucose in the intestine of Thyone, 387 (abstract).
Runnström, J. On some properties of the jelly coat in sea urchin oocytes and eggs, 132.
Rustad, R. C., S. Yuyama and L. C. Rustad. Nuclear-cytoplasmic relations in radiation sensitivity, 388 (abstract).

S

Salinity tolerances of estuarine planktonic crustaceans, 108.
Salt-absorbing cells in gills of Callinectes and Carcinus, 367 (abstract).
Salt regulation in crabs, 447.
Sanger, J. W., and A. G. Szent-Györgyi. Band pattern changes in the striated adductor muscle of Pecten, 391 (abstract).
Sato, H., and S. Inoué. Condensation of the sperm nucleus and alignment of DNA molecules during spermiogenesis in Loligo, 357 (abstract).
Schmeer, Sr. M. Rosaril. Chemical and biological characteristics of growth-inhibiting agents from Mercenaria extracts, 388 (abstract).
Schmidt-Koenig, K. Sun compass orientation of pigeons upon displacement north of the Arctic Circle, 154.
Schneiderman, H. A. See A. Krishnakumaran, 378 (abstract).
Schnitzler, R. M. See W. L. Wilson, 396 (abstract).
Schuel, R. See L. Lorand, 379 (abstract).
Scott, A. A new concept of the mechanism of cytokinesis, 389 (abstract).
Sea urchin eggs, effects of mercaptoethanol on furrowing capacity of, 345.
Sea urchin eggs and oocytes, jelly coat of, 132.
Sea urchin gut, histochemistry of, 280.
Seasonal regulation in dragonfly, 304.
Secretion of phenol red by Mustelus, 377 (abstract).
Seliger, H. H., J. B. Buck, W. G. Fastie and W. D. McElroy. Flash patterns in Jamaican fireflies, 159.
Senturia, J. B. See H. W. Harry, 271.
Sequence of phosphorylation changes in irradiated cellular particulates, 173.
Sequential induction of presumptive epidermis in Rana gastrula, 413.
Serum antibody synthesis in bullfrog larvae, 232.
Setogenesis in Petrolisthes, 97.
Sexual activities in Aedes, 324.
Sexual differences in metabolism of Pyrrhocoris, 499.
Sexual differences in salinity tolerances of crustaceans, 108.
Shedding substance of Asterias nerve extracts, in vitro studies on, 381 (abstract).
Sichel, F. J. M. Electrically excitable systems of voluntary muscle, 389 (abstract).
Silberman, L. See A. M. Zimmerman, 355 (abstract).
Silkworm, photoperiodic control of diapause in, 511.
Simon, E. J. See D. Van Praag, 394 (abstract).
Sláma, K. Hormonal control of respiratory metabolism during growth, reproduction, and diapause in male adults of Pyrrhocoris, 499.
Slime mold, effect of light on cell aggregation in, 85.
Smith, A. E. S. The localization of acid phosphatase in the eggs of several species of invertebrates, 389 (abstract).
Smith, W. R. Interactions between Limulus and two species of marine bacteria, 390 (abstract).
Snail behavior, effect of gases on, 271.
Sol-gel reactions in amoebae, effects of mercaptoethanol on, 538.
Speidel, C. C., and R. H. Cheney. Comparative deviations in motility of developing sea urchins induced by irradiation, 353 (abstract).
Sperm, effect of on sea urchin egg jelly coat, 132.

Sperm motility, method of rating, 363 (abstract).

Sperm senescence in Arbacia, 363 (abstract).

Spielman, A. The mechanics of copulation in Aedes, 324.

Spiochaetopterus, tube-building and feeding in, 397.

Spleen, rat, effect of gamma radiation on, 173, 526.

Splenomegaly of chick embryo, cell interaction in, 467.

Sponge cell aggregation, effect of inhibition of energy metabolism and protein synthesis on, 375 (abstract).

Sponge cell reaggregation, effects of temperature on, 364 (abstract).

Starvation, effect of on composition of Nephtys coelomic fluid, 63.

Stephens, R. E. Fluorescent antimyosin labeling in locally contracted chick myofibrils, 390 (abstract).

Stephens, R. E. Ultraviolet microbeam studies of contraction in invertebrate striated muscle, 391 (abstract).

Strength, geomagnetic, adaptation of magnetoreceptive mechanism of snails to, 221.

Strongylocentrotus gut, histochemistry of, 280.

Studies on the irradiation of cellular particulates. IV., 173; V., 526.

Succinic dehydrogenase activity in tubularian development, 380 (abstract).

Succinoxidase activity in Dugesia homogenates, 317.

Sulfated mucopolysaccharides of sea urchin gut, 280.

Sulfhydryl, effects of on cleavage of Arbacia eggs, 345.

Sullivan, D. Studies of the ribosomal proteins of Arbacia eggs, 392 (abstract).

Sun compass orientation of pigeons north of Arctic Circle, 154.

Swimbladder of fish, gas secretion in, 367 (abstract).

Swimbladder muscle of toadfish, mechanics of, 382 (abstract).

Syllid annelid, development of, 187.

Synthesis of serum antibody in bullfrog larvae, 232.

Szent-Györgyi, A. G. See J. W. Sanger, 391 (abstract).

T

Taphius behavior, effect of gases on, 271.

Taylor, W. R. The uptake of EDTA by pure cultures of marine plankton algae, 392 (abstract).

Taylor, W. R., and C. D. Gebelein. Chromatographic analyses of plant pigments in intertidal sediments, 393 (abstract).

Temora, salinity tolerances of, 108.

Temperature, effect of on mercaptoethanol-treated amoebae, 538.

Temperature, role of in control of diapause of Antherea, 511.

Temperature, role of in kinetics of gastropod ribonucleases, 489.

Temperature characteristics of excitation in squid axon, 372 (abstract).

Terrestrial habitat of crabs, relation of to water and salt regulation, 447.

Testes of apholate-treated mosquitoes, 119.

Testis mitochondria, rat, effects of gamma radiation on, 173, 526.

Tetragoneuria, life-history and photoperiodic responses of, 304.

Thermal environment, role of in temperature coefficients of gastropod ribonucleases, 489.

Thiol groups, importance of in division of marine eggs, 345.

Thiol groups, importance of in form and mobility of amoebae, 538.

Thymidine incorporation into Arbacia eggs under pressure, 355 (abstract).

Thymidine and uridine, H^3-labeled, incorporation of into Pectinaria oocytes, 394 (abstract).

Time sequence of phosphorylation changes in irradiated cellular particulates, 173.

Tolerances, salinity, of estuarine planktonic crustaceans, 108.

Torch, R. The effects of actinomycin D on head regeneration in a brackish-water ciliate, Tracheloraphis, 393 (abstract).

Transport and absorption of D-glucose by Thyone intestine, 387 (abstract).

Trends among crabs in salt regulation, 447.

Trochophore of Acmaea, 294.

Troll, W., L. Fishman, S. Jaffe and J. Chasis. DNA in Arbacia, 394 (abstract).

Tube-building in Spiochaetopterus, 397.

Turbellaria, marine, of Woods Hole region, 364 (abstract).

Tweedell, K. S. Incorporation of H^3-thymidine and H^3-uridine by oocytes of Pectinaria, 394 (abstract).

Tyrosine as precursor of Uca black pigment, 239.

U

Uca, color change in, 239.

Uca, water and salt regulation in, 447.

UEHARA, M. See T. HUMPHREYS, 375 (abstract).

V

VAISNYS, J. R. See A. NOVICK, 478.
VAN PRAAG, D., E. J. SIMON AND H. S. ROSENKRANZ. The effects of various bacterial and viral inhibitors on the development and metabolism of fertilized sea urchin eggs, 394 (abstract).
Veliger of Acmaea, 294.
VILLEE, C. A. See R. B. BILLIAR, 362 (abstract).
VUNAKIS, H. V. See L. LEVINE, 379 (abstract).

W

WALD, G. See D. BOWNDS, 359 (abstract).
WALSH, G. E. Seasonal and diurnal fluctuations in the quantity of dissolved carbohydrate in Oyster Pond, Cape Cod, 395 (abstract).
Water circulation in Spiochaetopterus, 397.
Water regulation in crabs, 447.
WATKINS, D., J. LEONARDS, P. K. DIXIT, S. J. COOPERSTEIN AND A. LAZAROW. Glucose stimulation of insulin release from toadfish islet tissue in vitro, 395 (abstract).
Wave conditions, effects of on sorting of shells, in swash zone, 353 (abstract).
WEBB, H. M. See F. A. BROWN, JR., 206, 221.
WIERCINSKI, F. J. See W. L. WILSON, 396 (abstract).
WILLIAMS, C. M., AND P. L. ADKISSON. Physiology of insect diapause. XIV., 511.
WILSON, W. L., F. J. WIERCINSKI AND R. M. SCHNITZLER. The effect of localized ultrasound on the isolated sartorius muscle fiber of frog, 396 (abstract).

Y

YOST, H. T., JR., R. M. GLICKMAN AND L. H. BECK. Studies on the effects of irradiation of cellular particulates. IV., 173.
YOST, H. T., JR., S. S. RICHMOND AND L. H. BECK. Studies on the effects of irradiation of cellular particulates. V., 526.
YUYAMA, S. See R. C. RUSTAD, 388 (abstract).

Z

VAN ZANDT, D. See M. R. CARRIKER, 365 (abstract).
ZIGMAN, S., J. MUNRO AND S. LERMAN. A cold-precipitable protein in the dogfish lens, 358 (abstract).
ZIGMAN, S. See S. LERMAN, 378 (abstract).
ZIMMERMAN, A. M. The effects of mercaptoethanol upon form and movement of Amoeba, 538.
ZIMMERMAN, A. M. Effects of mercaptoethanol on the furrowing capacity of Arbacia eggs, 345.
ZIMMERMAN, A. M., AND L. SILBERMAN. Further studies on incorporation of H^3 thymidine in Arbacia eggs under hydrostatic pressure, 355 (abstract).
ZULLO, V. A. Re-evaluation of the late Cenozoic cirriped, "Tamiosome," 360 (abstract).
ZWEIFACH, B. W. See A. JANOFF, 356 (abstract).

Volume 127 Number 1

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JAMES CASE, University of California, Santa Barbara
JOHN W. GOWEN, Iowa State College
SALLY HUGHES-SCHRADER, Duke University
LIBBIE H. HYMAN, American Museum of Natural History
SHINYA INOUÉ, Dartmouth College
J. LOGAN IRVIN, University of North Carolina
L. H. KLEINHOLZ, Reed College
JOHN H. LOCHHEAD, University of Vermont
ROBERTS RUGH, Columbia University
WM. RANDOLPH TAYLOR, University of Michigan
CARROLL M. WILLIAMS, Harvard University

DONALD P. COSTELLO, University of North Carolina
Managing Editor

AUGUST, 1964

Marine Biological Laboratory
LIBRARY
AUG 2 4 1964
WOODS HOLE, MASS.

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, footnotes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A *condensed title* or running head of no more than 35 letters and spaces should be included.

4. *Literature Cited.* The list of papers cited should conform exactly to the style set in a recent issue of The Biological Bulletin; this list should be headed LITERATURE CITED, and typed *double*-spaced on separate pages.

5. *Figures.* The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction, but not larger than 15 × 22 inches. Explanatory matter should be included in legends as far as possible, not lettered on the illustrations. Figures should be prepared for reproduction as line cuts or halftones; other methods will be used only at the author's expense. Figures to be reproduced as line cuts should be drawn in black ink on white paper, good quality tracing cloth or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on Bristol Board, and any designating numbers or letters should be made directly on the figures. *All* figures should be numbered in consecutive order, with no distinction between text- and plate-figures. The author's name should appear on the reverse side of all figures, as well as the desired reduction.

6. *Mailing.* Manuscripts should be packed flat. **All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.**

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $3.75. Subscription per volume (three issues), $9.00, (this is $18.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Copyright © 1964, by the Marine Biological Laboratory

Second-class postage paid at Lancaster, Pa.

MBL/WHOI LIBRARY
WH 1AZX %